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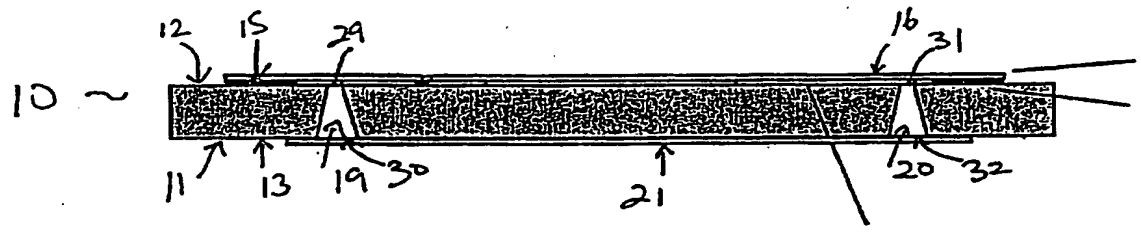

(57) Abstract: The present invention relates to an apparatus for performing biological reactions. Specifically, the invention relates to an apparatus for performing nucleic acid hybridization reactions on a substrate layer having a multiplicity of oligonucleotide binding sites disposed thereon, using substrates with flexible covers.

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COMPOSITIONS AND METHODS FOR PERFORMING BIOLOGICAL REACTIONS

This application is a continuing application of U.S.S.N. 09/492,013, filed January 26, 2000.

FIELD OF THE INVENTION

The present invention relates to an apparatus for performing biological reactions. In particular, the invention relates to an apparatus for performing nucleic acid hybridization reactions on a substrate layer having a multiplicity of oligonucleotide binding sites disposed thereon, using substrates with flexible covers, and a method for removing gas bubbles from the apparatus. Specifically, the invention relates to an apparatus having a flexible, gas permeable layer affixed to a substrate layer with an adhesive, wherein the flexible, gas permeable layer, the adhesive and the substrate layer enclose a reaction chamber, and a means for facilitating diffusion across the flexible, gas permeable layer. The diffusion-facilitating means creates a pressure gradient or concentration gradient across the flexible, gas permeable layer, thereby increasing the rate of diffusion of gas molecules from the reaction chamber across the flexible, gas permeable layer.

BACKGROUND OF THE INVENTION

Recent advances in molecular biology have provided the opportunity to identify pathogens, diagnose disease states, and perform forensic determinations using gene sequences specific for the desired purpose. This explosion of genetic information has created a need for high-capacity assays and equipment for performing molecular biological assays, particularly nucleic acid hybridization assays. Most urgently, there is a need to miniaturize, automate, standardize and simplify such assays. This need stems from the fact that while these hybridization assays were originally developed in research laboratories working with purified products and performed by highly skilled individuals, adapting these procedures to clinical uses, such as diagnostics, forensics and other applications, has produced the need for equipment and methods that allow less-skilled operators to effectively perform the assays under higher capacity, less stringent assay conditions.

Existing technology utilizes the binding of molecules contained within a biologically reactive sample

fluid, hereinafter referred to as target molecules, onto molecules contained within biologically reactive sites, hereinafter referred to as probe molecules. The primary enabler of this technology is an apparatus commonly referred to as a biochip, which comprises one or more ordered microscopic arrays ("microarrays") of biologically reactive sites immobilized on the surface of a substrate. A biologically reactive site can be created by dispensing a small volume of a fluid containing a biological reagent onto a discrete location on the surface of a substrate, also commonly referred to as spotting. To enhance immobilization of probe molecules, biochips can include a 2-dimensional array of 3-dimensional polymeric anchoring structures (for example, polyacrylamide gel pads) attached to the surface of the substrate. Probe molecules such as oligonucleotides are covalently attached to polyacrylamide-anchoring structures by forming amide, ester or disulfide bonds between the biomolecule and a derivatized polymer comprising the cognate chemical group. Covalent attachment of probe molecules to such polymeric anchoring structures is usually performed after polymerization and chemical cross-linking of the polymer to the substrate is completed.

Biochips are advantageously used to perform biological reactions on the surface thereof. Existing apparatus for performing biological reactions on a substrate surface, however, are deficient in that they either require unacceptably large volumes of sample fluid to operate properly, cannot accommodate substrates as large as or larger than a conventional microscope slide, cannot independently accommodate a plurality of independent reactions, or cannot accommodate a substrate containing hydrogel-based microarrays. Most existing apparatus also do not allow introduction of fluids in addition to the sample fluid (such as wash buffers, fluorescent dyes, etc.) into the reaction chamber. Disposable apparatus must be disassembled and reassembled around the biochip every time a new fluid must be introduced. Other existing apparatus are difficult to use in a laboratory environment because they cannot be loaded with standard pipet tips and associated pipettor apparatus.

Many existing apparatus also exhibit unacceptable reaction reproducibility, efficiency, and duration. Reaction reproducibility may be adversely affected by bubble formation in the reaction chamber or by the use of biologically incompatible materials for the reaction chamber. Reaction duration and efficiency may be adversely affected by the presence of concentration gradients in the reaction chamber.

Bubbles can form upon introduction of sample fluid to the reaction chamber or by outgassing of the reaction chamber materials. When gas bubbles extend over the substrate surface in an area containing biologically reactive sites, the intended reaction may intermittently fail or yield erroneous results because the intended concentration of the sample fluid mixture has been compromised by the presence of gas bubbles.

Biologically incompatible reaction chamber materials may cause unacceptable reaction reproducibility,

by interacting with the sample fluid, thus causing the intended reaction to intermittently fail or yield erroneous results.

Incomplete mixing of the sample fluid can introduce concentration gradients within the sample fluid that adversely impact reaction efficiency and duration. This effect is most pronounced when there is a depletion of target molecules in the local volume surrounding a biologically reactive site. During a biological reaction, the probability that a particular target molecule will bind to a complementary (immobilized) probe molecule is determined by the given concentration of target molecules present within the sample fluid volume, the diffusion rate of the target molecule through the reaction chamber, and the statistics of interaction between the target molecule and the complementary probe molecule. For diagnostic assays, target DNA molecules are often obtained in minute (< picomol) quantities. In practice, it can take tens of hours for a hybridization reaction to be substantially complete at the low target nucleic acid molecule levels available for biological samples. Concentration gradients in the hybridization chamber can further exacerbate this problem.

U.S. Patent 5,948,673 to Cottingham discloses a self-contained multi-chamber reactor for performing both DNA amplification and DNA probe assays in a sealed unit wherein some reactants are provided by coating the walls of the chambers and other reactants are introduced into the chambers prior to starting the reaction in order to eliminate flow into and out of the chamber. No provisions are made for eliminating gas bubbles from the chambers.

There remains a need in the art for methods and apparatus for performing biological reactions on a substrate surface that use a low volume of sample fluid, that accommodate substrates as large as or larger than a conventional microscope slide, that accommodate a plurality of independent reactions, and that accommodate a substrate surface having one or more hydrogel-based microarrays attached thereto. There also remains a need in the art for an apparatus that allows introduction of fluids in addition to sample fluid into each reaction chamber via standard pipet tips and associated pipettor apparatus. There also remains a need in the art for such an apparatus that increases reaction reproducibility, increases reaction efficiency, and reduces reaction duration. There also remains a need in this art for a simple method for removing gas bubbles from such an apparatus. These needs are particularly striking in view of the tremendous interest in biochip technology, the investment and substantial financial rewards generated by research into biochip technology, and the variety of products generated by such research.

Nucleic acid hybridization assays are advantageously performed using probe array technology, which utilizes binding of target single-stranded DNA onto immobilized DNA (usually, oligonucleotide) probes. The detection limit of a nucleic acid hybridization assay is determined by the sensitivity of the detection device, and also by the amount of target nucleic acid available to be bound to probes, typically oligonucleotide probes, during hybridization.

Nucleic acid hybridization chambers are known in the prior art. U.S. Patent No. 5,100,755 to Smyczek et al. discloses a hybridization chamber. U.S. Patent No. 5,545,531 to Rava et al. discloses a hybridization plate comprising a multiplicity of oligonucleotide arrays. U.S. Patent No. 5,360,741 to Hunnell discloses a gas heated hybridization chamber. U.S. Patent No. 5,922,591 to Anderson et al. discloses a miniaturized hybridization chamber for use with oligonucleotide arrays. U.S. Patent No. 5,945,334 to Besemer discloses oligonucleotide array packaging.

As currently employed, oligonucleotide array technology does not provide maximum hybridization efficiency. Existing nucleic acid hybridization assay equipment includes numerous components, each of which is a source of inefficiency and inaccuracy.

Hybridization using oligonucleotide arrays must be performed in a volume in which a small amount of target DNA or other nucleic acid can be efficiently annealed to the immobilized probes. For diagnostic assays, target DNA molecules are often obtained in minute (< picomol) quantities. In practice, it can take several (tens of) hours for hybridization to be substantially complete at the low target nucleic acid levels available for biological samples.

In addition, array hybridization is conventionally performed in a stationary hybridization chamber where active mixing is absent. Under these conditions, the probability that a particular target molecule will hybridize to a complementary oligonucleotide probe immobilized on a surface is determined by the concentration of the target, the diffusion rate of the target molecule and the statistics of interaction between the target and the complementary oligonucleotide. Consequently, a larger number of samples must be tested to obtain useful information, and this in turn leads to increased hybridization times and inefficiencies.

In addition, efficiency is increased when the amount of user manipulation is kept to a minimum. As currently performed, oligonucleotide array hybridization requires a great deal of operator attentiveness and manipulation, and the degree of skill required to perform the analysis is high. For example, hybridization is typically performed in an assay chamber, and then data collection and analysis are performed in a separate apparatus (such as a laser scanner or fluorescence microscope). This arrangement requires a substantial amount of handling by the user, and makes the assays both time-consuming and subject to user error.

It is also a limitation of current practice that array hybridizations are performed one array at a time, thereby forgoing the economies of parallel processing and data analysis.

Additional limitations, inefficiencies, and expenses arise from the structural characteristics of existing apparatus. Many existing apparatus are limited in the size of the substrate they can accommodate. Other apparatus are not disposable and therefore require extensive cleaning between runs in order to

prevent sample contamination. Yet other apparatus are high mass and therefore not susceptible of the rapid heating and cooling necessary for efficient hybridization. Other apparatus require the use of expensive optics for analysis of the reaction products.

There remains a need in this art for an easy-to-use apparatus for performing biological reactions, particularly nucleic acid hybridization, that comprises a small reaction volume, where the fluid components can be actively mixed, that can be performed in parallel and that minimizes user intervention. There also remains a need for such an apparatus that is easy to manufacture in various sizes, that is disposable to minimize sample contamination, that allows for the use of low cost optical analytical equipment, and that is low mass to allow for rapid heating and cooling of the sample fluid. There also remains a need for methods for using such apparatus to increase hybridization efficiency, particularly relating to biochip arrays as understood in the art. This need is particularly striking, in view of the tremendous interest in biochip technology, the investment and substantial financial rewards generated by research into biochip technology, and the variety of products generated by such research.

SUMMARY OF THE INVENTION

In accordance with the objects outlined above, the present invention provides apparatus for performing biological reactions, comprising a substrate that has a first and a second surface (usually opposite to each other, in the case of planar substrates). An array of biomolecules are positioned on the first surface, and a flexible layer affixed to the first surface of the substrate by an adhesive layer, wherein the adhesive layer is deposited on the first surface and forms a reaction volume. The substrate comprises at least a first port extending through the substrate from the first to the second surface, and the port has a first opening and a second opening. The first opening of the port is provided within the area bounded by the adhesive and covered by the flexible layer (e.g. the reaction volume) and the second opening of the port is provided on the second surface of the substrate. Alternatively, the port(s) may be in the flexible layer into the reaction volume.

Optionally, there may be a removable cover positioned over the second opening of the port such as a foil tape, as well as a layer of a water-soluble compound that is a solid at a first temperature and a liquid at a second, higher temperature, the layer being positioned between the first surface of the substrate and the flexible layer. Optionally, the flexible layer is a translucent plastic or gas permeable membrane.

In a further aspect, the invention provides an apparatus for performing biological reactions on a substrate surface and a method for removing gas bubbles from the apparatus to prevent interference with biological reactions such as hybridization at reaction sites on the substrate surface. Specifically, the method of the invention is directed to an apparatus comprising a flexible, gas permeable layer affixed to a biochip with an adhesive, wherein the flexible, gas permeable layer, the adhesive, and the

biochip enclose a reaction chamber, and a means for facilitating diffusion of gas molecules out of the reaction chamber across the flexible, gas permeable layer. The diffusion-facilitating means, referred to herein as a gas diffusion accelerator, creates a pressure gradient or concentration gradient across the flexible, gas permeable layer, thereby increasing the rate of diffusion of gas molecules from the reaction chamber through the flexible, gas permeable layer.

The port can be in the shape of a truncated cone having a small-diameter end and a large diameter end, and wherein the small diameter end is provided in the area on the first substrate surface bounded by the adhesive layer (the reaction volume) and the large diameter end is provided on the second substrate surface. The walls of a port in some embodiments form an angle of less than 90° with the second substrate surface. Optionally, the port contains a sample fluid having a contact angle, and the angle formed between the second substrate surface and the port walls is less than or equal to the contact angle of the sample fluid.

A second port can be included; in this embodiment, the second port extends through the substrate from the first surface to the second surface thereof and having a first opening and a second opening, wherein the first opening of the outlet port is provided on the first substrate surface within the area bounded by the adhesive and covered by the flexible layer, and the second opening of the outlet port is provided on the second substrate surface and is covered by a removable cover. Alternatively, the second port is in the flexible layer as above.

Optionally, the apparatus of the invention can comprise a reflective layer positioned between the array and the first substrate surface, and/or a resistive heater; the latter can be a reflective layer positioned between the array and the first substrate surface.

Additionally, the apparatus can comprise a passivation layer such as parylene, or a scanner, wherein the scanner is in contact with the flexible layer at a position over the array. The scanner can be a light pipe and cover the entirety of the reaction volume.

In a further aspect, the apparatus further comprises a sample preparation chip in contact with the second substrate surface wherein the sample preparation chip has a port that is aligned with the first port of the apparatus.

In an additional aspect, the apparatus further comprises a roller, wherein the roller is in contact with the flexible layer at a position over the array; for example, it can move longitudinally across the reaction volume.

In a further aspect, the apparatus can include a case having a lid and a base with a cavity, and a carriage comprising a scanner and a roller, wherein the carriage is provided in the cavity, wherein the

substrate is removably positioned above the carriage such that the first substrate surface is in operative contact with the carriage.

In an additional aspect, the invention provides apparatus for performing biological reactions, comprising a glass microscope slide having a first surface and a second surface opposite thereto; an array of biomolecules positioned on the first surface of the slide, wherein each biomolecule within the array is anchored to the first surface by a polyacrylamide gel pad; a layer of polyvinylidene chloride affixed to the first surface of the slide by a layer of double-sided adhesive, wherein the adhesive layer is deposited on the first surface of the slide and encloses an area thereupon; a first and second port extending through the slide from the first surface to the second surface thereof each having a first opening and a second opening, wherein the first opening of each port is provided within the area on the first surface of the slide bounded by the adhesive and covered by the flexible layer and the second opening of each port is provided on the second surface of the slide, and wherein each port is in the shape of a truncated cone having a small-diameter end and a large diameter end, and wherein the small diameter end is the first opening and the large diameter end is the second opening; a layer of foil tape positioned over the second opening of each port; a layer of a polyethylene glycol positioned between the first surface of the slide and the layer of polyvinylidene chloride; a reflective layer positioned between the array and the first substrate surface; a layer of parylene positioned between the reflective layer and the and the layer of polyvinylidene chloride; and a resistive heater.

In a further aspect, the invention provides an apparatus for performing biological reactions, comprising a substrate having a first surface and a second surface, a multiplicity of biomolecules positioned on the first surface of the substrate, a flexible layer affixed to the first surface of the substrate by an adhesive layer, wherein the adhesive layer is deposited on the first surface of the substrate and encloses an area thereupon, and wherein a reaction volume is enclosed between the flexible layer and the first substrate surface in the area defined by the adhesive layer. There are first and second ports extending through the flexible layer and the adhesive layer into the volume enclosed between the flexible layer and the first substrate surface in the area defined by the adhesive layer.

In an additional aspect, each of the multiplicity of biomolecules is attached to an anchoring structure such as a gel pad of a polymeric material such as polyacrylamide, either as discrete sites or as a continuous layer.

In a further aspect, the apparatus comprises a label layer affixed to the flexible layer, wherein the first and second ports extend through the label layer and the flexible layer. The label layer can comprise an adhesive surface and a non-adhesive surface, and wherein the label layer is affixed to the flexible layer using the adhesive surface. The label layer can comprise a window corresponding in size and position to the area bounded by the adhesive layer, and wherein the window allows visual inspection of the flexible layer and the volume enclosed between the flexible layer and the first substrate surface in

the area defined by the adhesive layer. Optionally, a reflective layer positioned between the array and the first substrate surface is provided, and/or a resistive heater.

In an additional aspect, the invention provides methods of using the apparatus to detect target analytes.

BRIEF DESCRIPTION OF THE DRAWINGS

Presently preferred embodiments of the invention are described with reference to the following drawings.

FIGS. **1A-1D** are views of a preferred embodiment of the present invention illustrating the preparation of a chamber for reaction. FIG. **1A** is a cross-sectional view of the apparatus illustrating a reaction chamber prefilled with a water-soluble compound in thermal contact with a heating element. FIG. **1B** is a cross sectional view of the apparatus illustrating the mixing of the water-soluble compound and the biological sample fluid. FIG. **1C** is a cross sectional view of the apparatus illustrating a chamber filled with the sample fluid/water-soluble compound mixture, wherein the first and second ports are covered with a seal. FIG. **1D** is a top plan view of the apparatus illustrating the pattern of adhesive defining the individual areas containing the arrays of oligonucleotide probes.

FIG. **2** is an exploded cross-sectional view of a chamber showing the array of gel pads of a preferred embodiment of the invention.

FIG. **3** is an exploded perspective view of the array of biomolecular probes showing the positioning of the gel pads on the substrate of a preferred embodiment of the invention.

FIG. **4** is an exploded cross-sectional view of a port illustrating the conical shape of the port of a preferred embodiment of the invention.

FIG. **5** is a perspective view of the label layer, the flexible layer and the adhesive layer of a preferred embodiment of the invention.

FIG. **6** is a cross-sectional view of a stack of chambers according to a preferred embodiment.

FIGS. **7A-7E** are top views of the layers of an alternate preferred embodiment of the invention having inlet and outlet ports extending through the flexible layer. FIG. **7A** is a view of the first adhesive layer, FIG. **7B** is a view of the flexible layer, FIG. **7C** is a view of the second adhesive layer, FIG. **7D** is a view of the label layer, and FIG. **7E** is a view of the layers of **7A** to **7D** as assembled.

FIGS. **8A-8B** are detail views of the notches cut into the first adhesive layer and the label layer of a preferred embodiment of the invention having inlet and outlet ports extending through the flexible layer.

FIGS. **9A-9C** are cross-sectional views of a preferred embodiment of the present invention illustrating the process of analyzing the array after completion of the reaction. FIG. **9A** shows the apparatus upon completion of the reaction. FIG. **9B** illustrates removal of the sample fluid from the chamber such that the flexible layer contacts the array. FIG. **9C** illustrates use of a laser scanner to analyze the array.

FIGS. **10A-10C** illustrate a handheld embodiment of the present invention. FIG. **10A** is a side view of the hand held scanning system. FIG. **10B** is a perspective view of a preferred embodiment comprising a hand-held scanning device illustrating the contact of the flexible layer with the carriage. FIG. **10C** is a view of the handheld system illustrating the lid containing the display unit.

FIG. **11A-11E** are cross-sectional views of the direct contact fiber optic scanner as shown in FIG. **10**.

FIG **12A-12C** are alternate embodiments illustrating the apparatus coupled to a sample preparation chip. FIG. **12A** illustrates an embodiment wherein the sample preparation chip is removably positioned against the second surface of the substrate. FIG. **12B** illustrates an embodiment wherein the sample preparation chip is affixed to the second surface of the substrate. FIG. **12C** illustrates an embodiment wherein the sample preparation chip is incorporated into the substrate.

FIG. **13** illustrates the assembly and use of a preferred embodiment of the present invention.

Figure 14 depicts a cross sectional view of a preferred embodiment of the present invention illustrating the application of vacuum to a reaction chamber or volume.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to methods and compositions for performing high capacity biological reactions on a biochip comprising a substrate having an array of biological binding sites. The invention provides a reaction chamber, such as a reaction chamber in the case where the biochip comprises nucleic acids. The reaction chamber is formed with a substrate, a layer of adhesive and a flexible cover. The system utilizes ports, either in the substrate or in the flexible cover, to allow sample and/or reagent loading. In addition, the invention provides methods for removing gas bubbles from the apparatus using a gas diffusion accelerator, that will facilitate and accelerate the rate of diffusion through the gas permeable, flexible membrane.

Accordingly, the present invention provides devices of the invention are used to detect target analytes

in samples. By "target analyte" or "analyte" or grammatical equivalents herein is meant any molecule, compound or particle to be detected. As outlined below, target analytes preferably bind to binding ligands, as is more fully described above. As will be appreciated by those in the art, a large number of analytes may be detected using the present methods; basically, any target analyte for which a binding ligand, described herein, may be made may be detected using the methods of the invention.

Suitable analytes include organic and inorganic molecules, including biomolecules. In a preferred embodiment, the analyte may be an environmental pollutant (including pesticides, insecticides, toxins, etc.); a chemical (including solvents, polymers, organic materials, etc.); therapeutic molecules (including therapeutic and abused drugs, antibiotics, etc.); biomolecules (including hormones, cytokines, proteins, lipids, carbohydrates, cellular membrane antigens and receptors (neural, hormonal, nutrient, and cell surface receptors) or their ligands, etc); whole cells (including procaryotic (such as pathogenic bacteria) and eukaryotic cells, including mammalian tumor cells); viruses (including retroviruses, herpesviruses, adenoviruses, lentiviruses, etc.); and spores; etc. Particularly preferred analytes are environmental pollutants; nucleic acids; proteins (including enzymes, antibodies, antigens, growth factors, cytokines, etc); therapeutic and abused drugs; cells; and viruses.

In a preferred embodiment, the target analyte is a nucleic acid. By "nucleic acid" or "oligonucleotide" or grammatical equivalents herein means at least two nucleotides covalently linked together. A nucleic acid of the present invention will generally contain phosphodiester bonds, although in some cases, as outlined below, nucleic acid analogs are included that may have alternate backbones, comprising, for example, phosphoramide (Beaucage et al., Tetrahedron 49(10):1925 (1993) and references therein; Letsinger, J. Org. Chem. 35:3800 (1970); Sprinzl et al., Eur. J. Biochem. 81:579 (1977); Letsinger et al., Nucl. Acids Res. 14:3487 (1986); Sawai et al, Chem. Lett. 805 (1984), Letsinger et al., J. Am. Chem. Soc. 110:4470 (1988); and Pauwels et al., Chemica Scripta 26:141 91986)), phosphorothioate (Mag et al., Nucleic Acids Res. 19:1437 (1991); and U.S. Patent No. 5,644,048), phosphorodithioate (Briu et al., J. Am. Chem. Soc. 111:2321 (1989), O-methylphophoroamidite linkages (see Eckstein, Oligonucleotides and Analogues: A Practical Approach, Oxford University Press), and peptide nucleic acid backbones and linkages (see Egholm, J. Am. Chem. Soc. 114:1895 (1992); Meier et al., Chem. Int. Ed. Engl. 31:1008 (1992); Nielsen, Nature, 365:566 (1993); Carlsson et al., Nature 380:207 (1996), all of which are incorporated by reference). Other analog nucleic acids include those with positive backbones (Denpcy et al., Proc. Natl. Acad. Sci. USA 92:6097 (1995); non-ionic backbones (U.S. Patent Nos. 5,386,023, 5,637,684, 5,602,240, 5,216,141 and 4,469,863; Kiedrowshi et al., Angew. Chem. Intl. Ed. English 30:423 (1991); Letsinger et al., J. Am. Chem. Soc. 110:4470 (1988); Letsinger et al., Nucleoside & Nucleotide 13:1597 (1994); Chapters 2 and 3, ASC Symposium Series 580, "Carbohydrate Modifications in Antisense Research", Ed. Y.S. Sanghui and P. Dan Cook; Mesmaeker et al., Bioorganic & Medicinal Chem. Lett. 4:395 (1994); Jeffs et al., J. Biomolecular NMR 34:17 (1994); Tetrahedron Lett. 37:743 (1996)) and non-ribose backbones, including those described in U.S. Patent Nos. 5,235,033 and 5,034,506, and

Chapters 6 and 7, ASC Symposium Series 580, "Carbohydrate Modifications in Antisense Research", Ed. Y.S. Sanghui and P. Dan Cook. Nucleic acids containing one or more carbocyclic sugars are also included within the definition of nucleic acids (see Jenkins et al., Chem. Soc. Rev. (1995) pp169-176). Several nucleic acid analogs are described in Rawls, C & E News June 2, 1997 page 35. Nucleic acid analogs also include "locked nucleic acids". All of these references are hereby expressly incorporated by reference. These modifications of the ribose-phosphate backbone may be done to facilitate the addition of electron transfer moieties, or to increase the stability and half-life of such molecules in physiological environments.

As will be appreciated by those in the art, all of these nucleic acid analogs may find use in the present invention. In addition, mixtures of naturally occurring nucleic acids and analogs can be made; for example, at the site of conductive oligomer or electron transfer moiety attachment, an analog structure may be used. Alternatively, mixtures of different nucleic acid analogs, and mixtures of naturally occurring nucleic acids and analogs may be made.

As outlined herein, the nucleic acids may be single stranded or double stranded, as specified, or contain portions of both double stranded or single stranded sequence. The nucleic acid may be DNA, both genomic and cDNA, RNA or a hybrid, where the nucleic acid contains any combination of deoxyribo- and ribo-nucleotides, and any combination of bases, including uracil, adenine, thymine, cytosine, guanine, inosine, xathanine hypoxathanine, isocytosine, isoguanine, etc. As used herein, the term "nucleoside" includes nucleotides and nucleoside and nucleotide analogs, and modified nucleosides such as amino modified nucleosides. In addition, "nucleoside" includes non-naturally occuring analog structures. Thus for example the individual units of a peptide nucleic acid, each containing a base, are referred to herein as nucleosides.

In a preferred embodiment, the present invention provides methods of detecting target nucleic acids. By "target nucleic acid" or "target sequence" or grammatical equivalents herein means a nucleic acid sequence on a single strand of nucleic acid. The target sequence may be a portion of a gene, a regulatory sequence, genomic DNA, cDNA, RNA including mRNA and rRNA, or others. It may be any length, with the understanding that longer sequences are more specific. In some embodiments, it may be desirable to fragment or cleave the sample nucleic acid into fragments of 100 to 10,000 basepairs, with fragments of roughly 500 basepairs being preferred in some embodiments. As will be appreciated by those in the art, the complementary target sequence may take many forms. For example, it may be contained within a larger nucleic acid sequence, i.e. all or part of a gene or mRNA, a restriction fragment of a plasmid or genomic DNA, among others.

As is outlined more fully below, probes (including primers) are made to hybridize to target sequences to determine the presence or absence of the target sequence in a sample. Generally speaking, this term will be understood by those skilled in the art.

The target sequence may also be comprised of different target domains, which may be adjacent (i.e. contiguous) or separated. For example, when ligation chain reaction (LCR) techniques are used, a first primer may hybridize to a first target domain and a second primer may hybridize to a second target domain; either the domains are adjacent, or they may be separated by one or more nucleotides, coupled with the use of a polymerase and dNTPs, as is more fully outlined below. The terms "first" and "second" are not meant to confer an orientation of the sequences with respect to the 5'-3' orientation of the target sequence. For example, assuming a 5'-3' orientation of the complementary target sequence, the first target domain may be located either 5' to the second domain, or 3' to the second domain.

In a preferred embodiment, the target analyte is a protein. As will be appreciated by those in the art, there are a large number of possible proteinaceous target analytes that may be detected using the present invention. By "proteins" or grammatical equivalents herein is meant proteins, oligopeptides and peptides, derivatives and analogs, including proteins containing non-naturally occurring amino acids and amino acid analogs, and peptidomimetic structures. The side chains may be in either the (R) or the (S) configuration. In a preferred embodiment, the amino acids are in the (S) or L-configuration. As discussed below, when the protein is used as a binding ligand, it may be desirable to utilize protein analogs to retard degradation by sample contaminants.

Suitable protein target analytes include, but are not limited to, (1) immunoglobulins, particularly IgEs, IgGs and IgMs, and particularly therapeutically or diagnostically relevant antibodies, including but not limited to, for example, antibodies to human albumin, apolipoproteins (including apolipoprotein E), human chorionic gonadotropin, cortisol, α-fetoprotein, thyroxin, thyroid stimulating hormone (TSH), antithrombin, antibodies to pharmaceuticals (including antieptileptic drugs (phenytoin, primidone, carbariezepin, ethosuximide, valproic acid, and phenobarbitol), cardioactive drugs (digoxin, lidocaine, procainamide, and disopyramide), bronchodilators (theophylline), antibiotics (chloramphenicol, sulfonamides), antidepressants, immunosuppresants, abused drugs (amphetamine, methamphetamine, cannabinoids, cocaine and opiates) and antibodies to any number of viruses (including orthomyxoviruses, (e.g. influenza virus), paramyxoviruses (e.g respiratory syncytial virus, mumps virus, measles virus), adenoviruses, rhinoviruses, coronaviruses, reoviruses, togaviruses (e.g. rubella virus), parvoviruses, poxviruses (e.g. variola virus, vaccinia virus), enteroviruses (e.g. poliovirus, coxsackievirus), hepatitis viruses (including A, B and C), herpesviruses (e.g. Herpes simplex virus, varicella-zoster virus, cytomegalovirus, Epstein-Barr virus), rotaviruses, Norwalk viruses, hantavirus, arenavirus, rhabdovirus (e.g. rabies virus), retroviruses (including HIV, HTLV-I and -II), papovaviruses (e.g. papillomavirus), polyomaviruses, and picornaviruses, and the like), and bacteria (including a wide variety of pathogenic and non-pathogenic prokaryotes of interest including Bacillus; Vibrio, e.g. *V. cholerae;* Escherichia, e.g. Enterotoxigenic *E. coli*, Shigella, e.g. *S. dysenteriae;* Salmonella, e.g. *S. typhi;* Mycobacterium e.g. *M. tuberculosis, M. leprae*; Clostridium, e.g. *C. botulinum, C. tetani, C. difficile, C.perfringens*; Cornyebacterium, e.g. *C. diphtheriae*;

Streptococcus, *S. pyogenes, S. pneumoniae;* Staphylococcus, *e.g. S. aureus*; Haemophilus, e.g. *H. influenzae*; Neisseria, e.g. *N. meningitidis, N. gonorrhoeae*; Yersinia, e.g. *G. lambliaY. pestis,* Pseudomonas, e.g. *P. aeruginosa, P. putida*; Chlamydia, e.g. *C. trachomatis*; Bordetella, e.g. *B. pertussis;* Treponema, e.g. *T. palladium;* and the like); (2) enzymes (and other proteins), including but not limited to, enzymes used as indicators of or treatment for heart disease, including creatine kinase, lactate dehydrogenase, aspartate amino transferase, troponin T, myoglobin, fibrinogen, cholesterol, triglycerides, thrombin, tissue plasminogen activator (tPA); pancreatic disease indicators including amylase, lipase, chymotrypsin and trypsin; liver function enzymes and proteins including cholinesterase, bilirubin, and alkaline phosphotase; aldolase, prostatic acid phosphatase, terminal deoxynucleotidyl transferase, and bacterial and viral enzymes such as HIV protease; (3) hormones and cytokines (many of which serve as ligands for cellular receptors) such as erythropoietin (EPO), thrombopoietin (TPO), the interleukins (including IL-1 through IL-17), insulin, insulin-like growth factors (including IGF-1 and -2), epidermal growth factor (EGF), transforming growth factors (including TGF-α and TGF-β), human growth hormone, transferrin, epidermal growth factor (EGF), low density lipoprotein, high density lipoprotein, leptin, VEGF, PDGF, ciliary neurotrophic factor, prolactin, adrenocorticotropic hormone (ACTH), calcitonin, human chorionic gonadotropin, cotrisol, estradiol, follicle stimulating hormone (FSH), thyroid-stimulating hormone (TSH), leutinzing hormone (LH), progeterone and testosterone; and (4) other proteins (including α-fetoprotein, carcinoembryonic antigen CEA, cancer markers, etc.).

In addition, any of the biomolecules for which antibodies may be detected may be detected directly as well; that is, detection of virus or bacterial cells, therapeutic and abused drugs, etc., may be done directly.

Suitable target analytes include carbohydrates, including but not limited to, markers for breast cancer (CA15-3, CA 549, CA 27.29), mucin-like carcinoma associated antigen (MCA), ovarian cancer (CA125), pancreatic cancer (DE-PAN-2), prostate cancer (PSA), CEA, and colorectal and pancreatic cancer (CA 19, CA 50, CA242).

Suitable target analytes include metal ions, particularly heavy and/or toxic metals, including but not limited to, aluminum, arsenic, cadmium, selenium, cobalt, copper, chromium, lead, silver and nickel.

These target analytes may be present in any number of different sample types, including, but not limited to, bodily fluids including blood, lymph, saliva, vaginal and anal secretions, urine, feces, perspiration and tears, and solid tissues, including liver, spleen, bone marrow, lung, muscle, brain, etc.

Accordingly, the present invention provides devices for the detection of target analytes comprising a solid substrate. The solid substrate can be made of a wide variety of materials and can be configured

in a large number of ways, as is discussed herein and will be apparent to one of skill in the art. In addition, a single device may be comprises of more than one substrate; for example, there may be a "sample treatment" cassette that interfaces with a separate "detection" cassette; a raw sample is added to the sample treatment cassette and is manipulated to prepare the sample for detection, which is removed from the sample treatment cassette and added to the detection cassette. There may be an additional functional cassette into which the device fits; for example, a heating element which is placed in contact with the sample cassette to effect reactions such as PCR. In some cases, a portion of the substrate may be removable; for example, the sample cassette may have a detachable detection cassette, such that the entire sample cassette is not contacted with the detection apparatus. See for example U.S. Patent No. 5,603,351, PCT US96/17116, and "MULTILAYERED MICROFLUIDIC DEVICES FOR ANALYTE REACTIONS" filed in the PCT December 11, 2000, Serial No. PCT/US00/33499, hereby incorporated by reference.

The composition of the solid substrate will depend on a variety of factors, including the techniques used to create the device, the use of the device, the composition of the sample, the analyte to be detected, the size of the wells and microchannels, the presence or absence of electronic components, etc. Generally, the devices of the invention should be easily sterilizable as well.

In a preferred embodiment, the solid substrate can be made from a wide variety of materials including, but not limited to, silicon such as silicon wafers, silcon dioxide, silicon nitride, glass and fused silica, gallium arsenide, indium phosphide, aluminum, ceramics, polyimide, quartz, plastics, resins and polymers including polymethylmethacrylate, acrylics, polyethylene, polyethylene terepthalate, polycarbonate, polystyrene and other styrene copolymers, polypropylene, polytetrafluoroethylene, superalloys, zircaloy, steel, gold, silver, copper, tungsten, molybdeumn, tantalum, KOVAR, KEVLAR, KAPTON, MYLAR, brass, sapphire, etc. High quality glasses such as high melting borosilicate or fused silicas may be preferred for their UV transmission properties when any of the sample manipulation steps require light based technologies. In addition, as outlined herein, portions of the internal surfaces of the device may be coated with a variety of coatings as needed, to reduce non-specific binding, to allow the attachment of binding ligands, for biocompatibility, for flow resistance, etc.

In a preferred embodiment, the solid support comprises ceramic materials, such as are outlined in U.S.S.N.s 09/235,081; 09/337,086; 09/464,490; 09/492,013; 09/466,325; 09/460,281; 09/460,283; 09/387,691; 09/438,600; 09/506,178; and 09/458,534; all of which are expressly incorporated by reference in their entirety. In this embodiment, the devices are made from layers of green-sheet that have been laminated and sintered together to form a substantially monolithic structure. Green-sheet is a composite material that includes inorganic particles of glass, glass-ceramic, ceramic, or mixtures thereof, dispersed in a polymer binder, and may also include additives such as plasticizers and dispersants. The green-sheet is preferably in the form of sheets that are 50 to 250 microns thick. The

ceramic particles are typically metal oxides, such as aluminum oxide or zirconium oxide. An example of such a green-sheet that includes glass-ceramic particles is "AX951" that is sold by E.I. Du Pont de Nemours and Company. An example of a green-sheet that includes aluminum oxide particles is "Ferro Alumina" that is sold by Ferro Corp. The composition of the green-sheet may also be custom formulated to meet particular applications. The green-sheet layers are laminated together and then fired to form a substantially monolithic multilayered structure. The manufacturing, processing, and applications of ceramic green-sheets are described generally in Richard E. Mistler, "Tape Casting: The Basic Process for Meeting the Needs of the Electronics Industry," Ceramic Bulletin, vol. 69, no. 6, pp. 1022-26 (1990), and in U.S. Patent No. 3,991,029, which are incorporated herein by reference.

The method for fabricating devices (such as those depicted in Figures 27-30 as devices 100 and 200) begins with providing sheets of green-sheet that are preferably 50 to 250 microns thick. The sheets of green-sheet are cut to the desired size, typically 6 inches by 6 inches for conventional processing, although smaller or larger devices may be used as needed. Each green-sheet layer may then be textured using various techniques to form desired structures, such as vias, channels, or cavities, in the finished multilayered structure.

Various techniques may be used to texture a green-sheet layer. For example, portions of a green-sheet layer may be punched out to form vias or channels. This operation may be accomplished using conventional multilayer ceramic punches, such as the Pacific Trinetics Corp. Model APS-8718 Automated Punch System. Instead of punching out part of the material, features, such as channels and wells may be embossed into the surface of the green-sheet by pressing the green-sheet against an embossing plate that has a negative image of the desired structure. Texturing may also be accomplished by laser tooling with a laser via system, such as the Pacific Trinetics LVS-3012.

Next, a wide variety of materials may be applied, preferably in the form of thick-film pastes, to each textured green-sheet layer. For example, electrically conductive pathways may be provided by depositing metal-containing thick-film pastes onto the green-sheet layers. Thick-film pastes typically include the desired material, which may be either a metal or a dielectric, in the form of a powder dispersed in an organic vehicle, and the pastes are designed to have the viscosity appropriate for the desired deposition technique, such as screen-printing. The organic vehicle may include resins, solvents, surfactants, and flow-control agents. The thick-film paste may also include a small amount of a flux, such as a glass frit, to facilitate sintering. Thick-film technology is further described in J.D. Provance, "Performance Review of Thick Film Materials," *Insulation/Circuits* (April, 1977) and in Morton L. Topfer, *Thick Film Microelectronics, Fabrication, Design, and Applications* (1977), pp. 41-59, which are incorporated herein by reference.

The porosity of the resulting thick-film can be adjusted by adjusting the amount of organic vehicle present in the thick-film paste. Specifically, the porosity of the thick-film can be increased by

increased the percentage of organic vehicle in the thick-film paste. Similarly, the porosity of a green-sheet layer can be increased by increasing the proportion of organic binder. Another way of increasing porosity in thick-films and green-sheet layers is to disperse within the organic vehicle, or the organic binder, another organic phase that is not soluble in the organic vehicle. Polymer microspheres can be used advantageously for this purpose.

To add electrically conductive pathways, the thick film pastes typically include metal particles, such as silver, platinum, palladium, gold, copper, tungsten, nickel, tin, or alloys thereof. Silver pastes are preferred. Examples of suitable silver pastes are silver conductor composition numbers 7025 and 7713 sold by E.I. Du Pont de Nemours and Company.

The thick-film pastes are preferably applied to a green-sheet layer by screen-printing. In the screen-printing process, the thick-film paste is forced through a patterned silk screen so as to be deposited onto the green-sheet layer in a corresponding pattern. Typically, the silk screen pattern is created photographically by exposure to a mask. In this way, conductive traces may be applied to a surface of a green-sheet layer. Vias present in the green-sheet layer may also be filled with thick-film pastes. If filled with thick-filled pastes containing electrically conductive materials, the vias can serve to provide electrical connections between layers.

After the desired structures are formed in each layer of green-sheet, preferably a layer of adhesive is applied to either surface of the green-sheet. Preferably, the adhesive is a room-temperature adhesive. Such room-temperature adhesives have glass transition temperatures below room temperature, *i.e.*, below about 20° C, so that they can bind substrates together at room temperature. Moreover, rather than undergoing a chemical change or chemically reacting with or dissolving components of the substrates, such room-temperature adhesives bind substrates together by penetrating into the surfaces of the substrates. Sometimes such room-temperature adhesives are referred to as "pressure-sensitive adhesives." Suitable room-temperature adhesives are typically supplied as water-based emulsions and are available from Rohm and Haas, Inc. and from Air Products, Inc. For example, a material sold by Air Products, Inc. as "Flexcryl 1653" has been found to work well.

The room-temperature adhesive may be applied to the green-sheet by conventional coating techniques. To facilitate coating, it is often desirable to dilute the supplied pressure-sensitive adhesive in water, depending on the coating technique used and on the viscosity and solids loading of the starting material. After coating, the room-temperature adhesive is allowed to dry. The dried thickness of the film of room-temperature adhesive is preferably in the range of 1 to 10 microns, and the thickness should be uniform over the entire surface of the green-sheet. Film thicknesses that exceed 15 microns are undesirable. With such thick films of adhesive voiding or delamination can occur during firing, due to the large quantity of organic material that must be removed. Films that are less

than about 0.5 microns thick when dried are too thin because they provide insufficient adhesion between the layers.

From among conventional coating techniques, spin-coating and spraying are the preferred methods. If spin-coating is used, it is preferable to add 1 gram of deionized water for every 10 grams of "Flexcryl 1653." If spraying is used, a higher dilution level is preferred to facilitate ease of spraying. Additionally, when room-temperature adhesive is sprayed on, it is preferable to hold the green-sheet at an elevated temperature, e.g., about 60 to 70° C, so that the material dries nearly instantaneously as it is deposited onto the green-sheet. The instantaneous drying results in a more uniform and homogeneous film of adhesive.

After the room-temperature adhesive has been applied to the green-sheet layers, the layers are stacked together to form a multilayered green-sheet structure. Preferably, the layers are stacked in an alignment die, so as to maintain the desired registration between the structures of each layer. When an alignment die is used, alignment holes must be added to each green-sheet layer. Typically, the stacking process alone is sufficient to bind the green-sheet layers together when a room-temperature adhesive is used. In other words, little or no pressure is required to bind the layers together. However, in order to effect a more secure binding of the layers, the layers are preferably laminated together after they are stacked.

The lamination process involves the application of pressure to the stacked layers. For example, in the conventional lamination process, a uniaxial pressure of about 1000 to 1500 psi is applied to the stacked green-sheet layers that is then followed by an application of an isostatic pressure of about 3000 to 5000 psi for about 10 to 15 minutes at an elevated temperature, such as 70° C. Adhesives do not need to be applied to bind the green-sheet layers together when the conventional lamination process is used.

However, pressures less than 2500 psi are preferable in order to achieve good control over the dimensions of such structures as internal or external cavities and channels. Even lower pressures are more desirable to allow the formation of larger structures, such as cavities and channels. For example, if a lamination pressure of 2500 psi is used, the size of well-formed internal cavities and channels is typically limited to no larger than roughly 20 microns. Accordingly, pressures less than 1000 psi are more preferred, as such pressures generally enable structures having sizes greater than about 100 microns to be formed with some measure of dimensional control. Pressures of less than 300 psi are even more preferred, as such pressures typically allow structures with sizes greater than 250 microns to be formed with some degree of dimensional control. Pressures less than 100 psi, which are referred to herein as "near-zero pressures," are most preferred, because at such pressures few limits exist on the size of internal and external cavities and channels that can be formed in the multilayered structure.

The pressure is preferably applied in the lamination process by means of a uniaxial press.

Alternatively, pressures less than about 100 psi may be applied by hand.

As with semiconductor device fabrication, many devices may be present on each sheet.

Accordingly, after lamination the multilayered structure may be diced using conventional green-sheet dicing or sawing apparatus to separate the individual devices. The high level of peel and shear resistance provided by the room-temperature adhesive results in the occurrence of very little edge delamination during the dicing process. If some layers become separated around the edges after dicing, the layers may be easily re-laminated by applying pressure to the affected edges by hand, without adversely affecting the rest of the device.

The final processing step is firing to convert the laminated multilayered green-sheet structure from its "green" state to form the finished, substantially monolithic, multilayered structure. The firing process occurs in two important stages as the temperature is raised. The first important stage is the binder burnout stage that occurs in the temperature range of about 250 to 500° C, during which the other organic materials, such as the binder in the green-sheet layers and the organic components in any applied thick-film pastes, are removed from the structure.

In the next important stage, the sintering stage, which occurs at a higher temperature, the inorganic particles sinter together so that the multilayered structure is densified and becomes substantially monolithic. The sintering temperature used depends on the nature of the inorganic particles present in the green-sheet. For many types of ceramics, appropriate sintering temperatures range from about 950 to about 1600° C, depending on the material. For example, for green-sheet containing aluminum oxide, sintering temperatures between 1400 and 1600° C are typical. Other ceramic materials, such as silicon nitride, aluminum nitride, and silicon carbide, require higher sintering temperatures, namely 1700 to 2200° C. For green-sheet with glass-ceramic particles, a sintering temperature in the range of 750 to 950° C is typical. Glass particles generally require sintering temperatures in the range of only about 350 to 700° C. Finally, metal particles may require sintering temperatures anywhere from 550 to 1700° C, depending on the metal.

Typically, the devices are fired for a period of about 4 hours to about 12 hours or more, depending on the material used. Generally, the firing should be of a sufficient duration so as to remove the organic materials from the structure and to completely sinter the inorganic particles. In particular, polymers are present as a binder in the green-sheet and in the room-temperature adhesive. The firing should be of sufficient temperature and duration to decompose these polymers and to allow for their removal from the multilayered structure.

Typically, the multilayered structure undergoes a reduction in volume during the firing process. During the binder burnout phase, a small volume reduction of about 0.5 to 1.5% is normally observed. At higher temperatures, during the sintering stage, a further volume reduction of about 14 to 17% is typically observed.

The volume change due to firing, on the other hand, can be controlled. In particular, to match volume changes in two materials, such as green-sheet and thick-film paste, one should match: (1) the particle sizes; and (2) the percentage of organic components, such as binders, which are removed during the firing process. Additionally, volume changes need not be matched exactly, but any mismatch will typically result in internal stresses in the device. But symmetrical processing, placing the identical material or structure on opposite sides of the device can, to some extent, compensate for shrinkage mismatched materials. Too great a mismatch in either sintering temperatures or volume changes may result in defects in or failure of some or all of the device. For example, the device may separate into its individual layers, or it may become warped or distorted.

As noted above, preferably any dissimilar materials added to the green-sheet layers are co-fired with them. Such dissimilar materials could be added as thick-film pastes or as other green-sheet layers, or added later in the fabrication process, after sintering. The benefit of co-firing is that the added materials are sintered to the green-sheet layers and become integral to the substantially monolithic microfluidic device. However, to be co-fireable, the added materials should have sintering temperatures and volume changes due to firing that are matched with those of the green-sheet layers. Sintering temperatures are largely material-dependent, so that matching sintering temperatures simply requires proper selection of materials. For example, although silver is the preferred metal for providing electrically conductive pathways, if the green-sheet layers contain alumina particles, which require a sintering temperature in the range of 1400 to 1600° C, some other metal, such as platinum, must be used due to the relatively low melting point of silver (961° C).

Alternatively, the addition of other substrates or joining of two post-sintered pieces can be done using any variety of adhesive techniques, including those outlined herein. For example, two "halves" of a device can be glued or fused together. For example, a particular detection platform, reagent mixture such as a hydrogel or biological components that are not stable at high temperature can be sandwiched in between the two halves. Alternatively, ceramic devices comprising open channels or wells can be made, additional substrates or materials placed into the devices, and then they may be sealed with other materials.

A particularly preferred substrate is glass, such as a microscope slide.

In a preferred embodiment, the solid substrate is configured for handling a single sample that may contain a plurality of target analytes. That is, a single sample is added to the device and the sample

may either be aliquoted for parallel processing for detection of the analytes or the sample may be processed serially, with individual targets being detected in a serial fashion. In addition, samples may be removed periodically or from different locations for in line sampling.

In a preferred embodiment, the solid substrate is configured for handling multiple samples, each of which may contain one or more target analytes. In general, in this embodiment, each sample is handled individually; that is, the manipulations and analyses are done in parallel, with preferably no contact or contamination between them. Alternatively, there may be some steps in common; for example, it may be desirable to process different samples separately but detect all of the target analytes on a single detection platform.

Furthermore, in some embodiments, the substrate comprises a multiplicity of arrays, particularly nucleic acid arrays, which are contained in one or a plurality of reaction volumes (e.g. bounded by the adhesive and covered by the flexible layer).

In addition, it should be understood that while most of the discussion herein is directed to the use of generally planar substrates with microchannels and wells, other geometries can be used as well. For example, two or more planar substrates can be stacked to produce a three dimensional device, that can contain microchannels flowing within one plane or between planes; similarly, wells may span two or more substrates to allow for larger sample volumes. Thus for example, both sides of a substrate can be etched to contain microchannels; see for example U.S. Patent Nos. 5,603,351 and 5,681,484, both of which are hereby incorporated by reference.

The biochip substrates of the invention have capture binding ligands attached in array formats. By "array" or "biochip" herein is meant a plurality of capture binding ligands, preferably nucleic acids, in an array format; the size of the array will depend on the composition and end use of the array. Most of the discussion herein is directed to the use of nucleic acid arrays with attached capture probes, but this is not meant to limit the scope of the invention, as other types of capture binding ligands (proteins, etc.), can be used. "Array" in this context generally refers to an ordered spatial arrangement, particularly an arrangement of immobilized biomolecules or polymeric anchoring structures. "Addressble array" refers to an array wherein the individual elements have precisely defined X and Y coordinates, so that a given element at a particular position in the array can be identified.

Nucleic acids arrays are known in the art, and can be classified in a number of ways; both ordered arrays (e.g. the ability to resolve chemistries at discrete sites), and random arrays are included. Ordered arrays include, but are not limited to, those made using photolithography techniques (Affymetrix GeneChip™), spotting techniques (Synteni and others), printing techniques (Hewlett Packard and Rosetta), three dimensional "gel pad" arrays, etc. The size of the array can vary; with arrays containing from about 2 different capture probes to many millions can be made, with very large

arrays being possible. Generally, the array will comprise from two to as many as 100,000, with from about 400 to about 1000 being the most preferred, and about 10,000 being especially preferred. Arrays can also be classifed as "addressable", which means that the individual elements of the array have precisely defined x and y coordinates, so that a given array element can be pinpointed.

The invention is advantageously used for performing assays using biochips **18**. Biochips, as used in the art, encompass substrates containing arrays or microarrays, preferably ordered arrays and most preferably ordered, addressable arrays, of biological molecules that comprise one member of a biological binding pair. Typically, such arrays are oligonucleotide arrays comprising a nucleotide sequence that is complementary to at least one sequence expected to be present in a biological sample. Alternatively, peptides or other small molecules can be arrayed in such biochips for performing immunological analysis (wherein the arrayed molecules are antigens) or assaying biological receptors (wherein the arrayed molecules are ligands, agonists or antagonists of said receptors). Thus, while "probes" generally refer to nucleic acids that are substantially complementary to target nucleic acids, "probe" and "biomolecular probe" can also refer to a biomolecule used to detect another biomolecule, e.g. its binding partner.

One useful feature of biochips is the manner in which the arrayed biomolecules are attached to the surface of the biochip. Conventionally such procedures involve multiple reaction steps, often requiring chemical modification of the solid support itself. Even in embodiments comprising absorption matrices, such as hydrogels, present on a solid support, chemical modification of the gel polymer is necessary to provide a chemical functionality capable forming a covalent bond with the biomolecule. The efficiency of the attachment chemistry and strength of the chemical bonds formed are critical to the fabrication and ultimate performance of the microarray.

Polymeric hydrogels and gel pads are used as binding layers to adhere to surfaces biological molecules including, but not limited to, proteins, peptides, oligonucleotides, polynucleotides, and larger nucleic acid fragments. The oligonucleotide probes may be bound to the surface of a continuous layer of the hydrogel, or to an array of gel pads. The gel pads comprising biochips for use with the apparatus of the present invention are conveniently produced as thin sheets or slabs, typically by depositing a solution of acrylamide monomer, a crosslinker such methylene bisacrylamide, and a catalyst such as N, N, N', N' - tetramethylethylendiamine (TEMED) and an initiator such as ammonium persulfate for chemical polymerization, or 2,2-dimethoxy-2-phenyl-acetophone (DMPAP) for photopolymerization, in between two glass surfaces (e.g., glass plates or microscope slides) using a spacer to obtain the desired thickness of the polymeric gel. Generally, the acrylamide monomer and crosslinker are prepared in one solution of about 4-5% acrylamide (having an acrylamide/ bisacrylamide ratio of 19/1) in water/glycerol, with a nominal amount of initiator added. The solution is polymerized and crosslinked either by ultraviolet (UV) radiation (e.g., 254 nm for at least about 15 minutes, or other appropriate UV conditions, collectively termed "photopolymerization"), or by thermal

initiation at elevated temperature (e.g., typically at about 40° C). Following polymerization and crosslinking, the top glass slide is removed from the surface to uncover the gel. The pore size (and hence the "sieving properties") of the gel is controlled by changing the amount of crosslinker and the percent solids in the monomer solution. The pore size also can be controlled by changing the polymerization temperature.

In the fabrication of polyacrylamide embodiments of the polymeric hydrogel arrays of the invention (i.e., patterned gels) used as binding layers for biological molecules, the acrylamide solution typically is imaged through a mask during the UV polymerization/crosslinking step. The top glass slide is removed after polymerization, and the unpolymerized monomer is washed away (developed) with water, leaving a fine feature pattern of polyacrylamide hydrogel, which is used to produce the crosslinked polyacrylamide hydrogel pads. Further, in an application of lithographic techniques known in the semiconductor industry, light can be applied to discrete locations on the surface of a polyacrylamide hydrogel to activate these specified regions for the attachment of an oligonucleotide, an antibody, an antigen, a hormone, hormone receptor, a ligand or a polysaccharide on the surface (e.g., a polyacrylamide hydrogel surface) of a solid support (*see, for example,* International Application, Publication No. WO 91/07087, incorporated by reference).

For hydrogel-based arrays using polyacrylamide, biomolecules (such as oligonucleotides) are covalently attached by forming an amide, ester or disulfide bond between the biomolecule and a derivatized polymer comprising the cognate chemical group. Covalent attachment of the biomolecule to the polymer is usually performed after polymerization and chemical cross-linking of the polymer is completed.

Alternatively, oligonucleotides bearing 5'-terminal acrylamide modifications can be used that efficiently copolymerize with acrylamide monomers to form DNA-containing polyacrylamide copolymers (Rehman *et al.*, 1999, *Nucleic Acids Research* 27: 649-655). Using this approach, stable probe-containing layers can be fabricated on supports (e.g., microtiter plates and silanized glass) having exposed acrylic groups. This approach has made available the commercially marketed "Acrydite™" capture probes (available from Mosaic Technologies, Boston, MA). The Acrydite moiety is a phosporamidite that contains an ethylene group capable of free-radical copolymerization with acrylamide, and which can be used in standard DNA synthesizers to introduce copolymerizable groups at the 5' terminus of any oligonucleotide probe.

With reference to the illustration provided in Figure 1, the invention provides a hybridization chamber **10** comprising a biochip, which comprises a substrate **11** having a first surface **12** and a second surface **13** opposite thereto, and a flexible layer **16** affixed to the first substrate surface **12** by an adhesive layer **15**. On the first surface **12** is an area **14** bounded by adhesive layer **15** an completely covered by flexible layer **16**. Flexible layer **16**, adhesive layer **15**, and first substrate surface **12** further

define a reaction volume **25** (also sometimes referred to herein as a reaction chamber). The ratio of volume **25** to area **14** is preferably from about 0.025 mL/mm^2 to about 0.25 mL/mm^2, more preferably from about 0.1 mL/mm^2 to about 0.25 mL/mm^2, and most preferably from about 0.1 mL/mm^2 to about 0.2 mL/mm^2.

While the present invention includes reaction volumes defined by the substrate, the adhesive and the flexible layer, as will be appreciated by those in the art, there are a variety of ways that the reaction volume can be formed. For example, rather than have an adhesive (in form of a gasket, for example) serve to create the "walls" of the chamber, the substrate itself may be formed to form these walls. As will be appreciated by those in the art, a wide variety of other configurations are also possible.

As shown in Figure 3, between flexible layer **16** and first substrate surface **12** in area **14** is positioned a multiplicity of biomolecules. In a preferred embodiment, the multiplicity of biomolecules comprises an array **17** of biomolecules, which is preferably affixed to first substrate surface **12**. Array **17** preferably further comprises gel pads **22**. In an alternate preferred embodiment, array **17** is deposited on a continuous layer of polyacrylamide. Figure 2 provides an exploded cross-sectional view of a portion of array **17** illustrating the gel pads **22**. Each gel structure **22** is preferably cylindrical, most preferably having about a 113 micron diameter and about a 25 micron thickness. The distance between each site within each array **17** is most preferably about 300 microns.

An optional layer of a water-soluble compound **28** is included that is either solid or highly viscous at a first temperature, e.g. room temperature or storage temperatures, and a liquid or more viscous at a second, higher temperature. Preferred embodiments utilize compounds having a melting point of about 30 to about 60°C, more preferably of about 35 to about 50°C, and most preferably of about 35 to about 45°C is deposited in volume **25** bounded by first substrate surface **12**, flexible layer **16**, and adhesive layer **15**. Preferably, the water-soluble compound is biocompatible, does not stick to flexible layer **16**, and serves to prevent mechanical damage to gel pads **22**. This compound can comprise any number of materials, with polymers such as glycol polymers, dextrans, sugars and other carbohydrates being preferred. In a preferred embodiment, the compound is polyethylene glycol, most preferably polyethylene glycol 600. The compound **28** is deposited so that the entire volume **25**, with the exception of that portion of volume **25** occupied by array **17**, comprises compound **28**.

Array **17** can be positioned on surface **12** by providing markings, most preferably holes or pits in surface **12**, that act as fiducials or reference points on surface **12** for accurate placement of array **17**. The presence of said fiducials is particularly advantageous in embodiments comprising a multiplicity of arrays **17** in one or a multiplicity of areas **14** on surface **12**, wherein accurate placement of said arrays is required for proper spacing and orientation of the arrays in the reaction chamber.

In preferred embodiments, a first and second port **19** and **20** extend through flexible layer **16**, as

shown in Figure 7, although in some embodiments there is only a single port that serves as both the inlet and outlet port. The first port **19** serves as an input port and is positioned in flexible layer **16** so that the first opening **29** is provided within the area **14** (reaction chamber) bounded by adhesive layer **15** on first surface **12**. Second port **20** serves as an outlet port and is positioned in flexible layer **16** so that the first opening **31** opens within area **14** bounded by the adhesive layer **15** on the first surface **12**.

Input and output ports **19** and **20** are preferably shaped to accept a plastic pipette tip, most preferably a 10μL pipette tip or a 200μL pipette tip. In preferred embodiments, input and output ports **19** and **20** are generally in the shape of a truncated cone, as shown in Figure 4, wherein the end of the cone having the smaller diameter forms the first opening of each port **29** and **31**, respectively, and the end of the cone having the larger diameter forms the second opening of each port **30** and **32**, respectively. This shape creates a seal between the pipette tip and the port, enhances visibility of the port for operator accuracy and prevents protrusion of the pipette tip into volume **25**, thereby preventing potential damage to components therein, particularly the flexible, gas permeable layer **16**. In these embodiments, each port preferably has a diameter on second substrate surface **13** of from about 1.0 mm to about 2.0 mm, and a diameter on first substrate surface **12** of from about 0.3 mm to about 0.6 mm. The conical walls of ports **19** and **20** form an angle **54** with the second substrate surface **13**, which is preferably less than 90°. Most preferably, angle **54** is less than or equal to the contact angle **55** of the biological sample fluid **26**. Most preferably, angle **54** is equal to contact angle **55** such that the surface of the fluid in the port is flat. For aqueous solutions, this angle is about 60°. This geometric arrangement provides a port that tends not to leak, but instead wicks fluid into volume **25** so that the second substrate surface **13** is dry when replaceable cover **21** is applied.

The openings of ports **19** and **20** may be covered with a removable and replaceable cover **21**. In preferred embodiments, replaceable cover **21** is a stopper, a gasket, or tape, most preferably a foil tape.

In some of these embodiments, one or more first notches **70** are cut into the first adhesive layer **15** such that the first notches **70** are in direct communication with the area **14** on first substrate surface **12** bounded by the first adhesive layer **15**. Second notches **72** are cut into the flexible layer **16** in positions corresponding to the size and position of first notches **70** in adhesive layer **15**, thus forming one or more ports. In a particularly preferred embodiment, a ring of adhesive **74** is deposited around the perimeter of each second notch **72**, such that the inner perimeter of adhesive ring **74** is coextensive with the outer perimeter of second notch **72**. Preferably, first and second notches **70** and **72** are circular in shape, and have a diameter that is equal to the inner diameter of adhesive ring **74**. Preferably the inner diameter and outer diameter of adhesive ring **74** are selected to form a tight seal with the tip end of a pipette. In an alternate preferred embodiment, a second layer of adhesive **76** is deposited on the portions of flexible layer **16** not covering the area **14** on first substrate surface **12** and

not defining first and second ports **19** and **20**. In this embodiment, the apparatus further comprises a label layer **57** that is die cut in the same manner as the first adhesive layer **15** to form windows **58** that correspond in location to areas **14** on first substrate surface **12**, and which is applied to second adhesive layer **76**. In this embodiment, one or more third notches **78** are cut into second adhesive layer **76**, such that third notches **78** correspond in shape, size, and position to first and second notches **70** and **72**. Fourth notches **80**, having a shape and position corresponding to first, second and third notches **70, 72** and **78**, are cut into label layer **57**. The diameter of fourth notches **80** is preferably greater than the diameter of first, second and third notches **70, 72** and **78**, such that after the apparatus is assembled a portion of second adhesive layer **76** is exposed by fourth notch **80**. Preferably the exposed portion of second adhesive layer **76** corresponds to the shape and size of a pipette tip.

In alternative embodiments of the apparatus, first and second ports **19** and **20** extend through substrate **11**, rather than through flexible layer **16**. Illustrative embodiments are described in co-owned and co-pending U.S. Application No. 09/464,490, incorporated by reference herein. In preferred embodiments of the apparatus, area **14** on first substrate surface **12** is square or rectangular with two rounded edges at diagonally opposite corners of are **14** and two 90 degree angles at the remaining two diagonally opposite corners of area **14**. Preferably, when first and second ports **19** and **20** extend through flexible layer **16**, first notches **70** in first adhesive layer **15** are cut at the sharp edges of area **14**, as shown in Figure 7. These embodiments are particularly preferred as they comprise geometries that eliminate corners and therefore are useful in the prevention of bubble formation in area **14**.

Substrate **11** is fabricated from any solid supporting substance, including but not limited to plastics, metals, ceramics, and glasses. Most preferably, substrate **11** is made from silicon or glass (for accuracy and stiffness), molded plastics (which reduce cost of manufacture and thermal inertia), or ceramics (for the incorporation of microfluidic elements including integrated heating elements). Most preferably, the substrate is glass.

Adhesive layer **15** is prepared using an adhesive suitable for forming a water-tight bond between substrate **11** and flexible layer **16**, including, but not limited to, high temperature acrylics, rubber-based adhesives, and silicone-based adhesives. The shape of adhesive layer **15** is configured to contain array **17**. Adhesive layer **15** can be deposited on first substrate surface **12** in a pattern to produce area **14** in any desired shape, and is most preferably deposited to define an ellipsoid area **14**. Adhesive layer **15** can be deposited using inkjet printing or offset printing methods, or by die cutting the desired shapes from a sheet of adhesive material. In addition, a substantial portion of first surface **12** can be covered with adhesive and portions of the substrate that are not desired to retain adhesive properties can be hardened preferentially, for example, by ultraviolet curing. In these embodiments,

portions retaining adhesive properties can be defined using a mask and thereby retain adhesive properties necessary to affix flexible layer **16** to surface **12**. In embodiments using the die cut adhesive material, the adhesive material is preferably a doublesided adhesive tape, and more preferably a double sided adhesive tape having no carrier. Adhesive layer **15** is most preferably set down in a layer between 1 and 100 μm thick, more preferably between 25 and 50 μm thick, and most preferably about 50 μm thick.

Flexible layer **16** is made of any flexible solid substance, including but not limited to plastics, including polypropylene, polyethylene, and polyvinylidene chloride (sold commercially as Saran® wrap) plastics, rubbers, including silicone rubbers, high temperature polyesters, and porous Teflon®. Flexible layer **16** is preferably both deformable and biocompatible and preferably has low permeability to liquids in order to prevent evaporation of water from the volume contained between the flexible layer and the substrate. That is, preferred embodiments utilized flexible layers that are selectively permeable to gas but impermeable or substantially impermeable to liquid. Flexible layer **16** also preferably is optically clear and should be able to withstand temperatures of between 50 and 95°C for a period of between 8 and 12 hours without shrinkage. Flexible layer **16** preferably covers an area of from about 5 mm^2 to about 1400 mm^2, more preferably from about 5 mm^2 to about 600 mm^2, and most preferably from about 100 mm^2 to about 600 mm^2.

In a preferred embodiment, the flexible layer is a gas permeable membrane. Most preferably, flexible, gas permeable layer **16** is selected to have physical, chemical and mechanical properties such that the surface tension of sample fluid **26** prevents passage of the sample fluid through the pores of the membrane, while allowing passage of gas molecules across the flexible, gas permeable layer. Preferably, the pore size of flexible, gas permeable layer **16** is between 0.2 and 3.0 μm, more preferably between 0.2 and 1 μm, and most preferably about 0.2 μm. Flexible, gas permeable layer **16** also preferably is translucent and should be able to withstand temperatures of between 50°C and 95°C for a period of between 8 and 12 hours without shrinkage. In a preferred embodiment, the flexible, gas permeable layer is porous Teflon®. Membranes having these characteristics are available from Pall Specialty Materials

In preferred embodiments, as shown in Figure 5, the invention further comprises a label layer **57** that is die cut in the same manner as the adhesive to form windows **58** that correspond in location to areas **14** on first substrate surface **12**. The label layer is preferably a thick film having a layer of adhesive, and most preferably is an Avery laser label. The label layer is applied to the outer surface of the flexible layer, preferably by vacuum lamination. Areas **14** are preferably visible through windows **58** in label layer **57**.

In a preferred embodiment, the invention further provides a means for facilitating diffusion across the flexible, gas permeable layer; this is referred to herein as a "gas diffusion accelerator". The gas

diffusion accelerator is used to increase the rate of diffusion of gas bubbles from the reaction chamber across the flexible layer, as compared to the diffusion rate in the absence of the accelerator. The gas diffusion accelerator can take on a variety of configurations, but is preferably removably affixed to the flexible, gas permeable layer, or the label layer when present, in order to remove gas bubbles from the reaction chambers. The gas diffusion accelerator creates a pressure gradient or concentration gradient across flexible, gas permeable layer **16**, thereby increasing the rate of diffusion of gas molecules from the sample fluid **26** contained in volume **25** across flexible, gas permeable layer **26**. A preferred embodiment of the gas diffusion accelerator , wherein the gas diffusion accelerator creates a pressure gradient across flexible, gas permeable layer **16**, is shown in Figure 14. In this embodiment, a vacuum source **70** is removably affixed to flexible, gas permeable layer **16**. In preferred embodiments, vacuum source **70** comprises a vacuum pump **71**, a chamber seal **72** that completely surrounds area **14** and is removably affixed to flexible, gas permeable layer **16**, and a length of plastic tubing **73** connecting vacuum pump **71** to reducer **72**. The chamber seal may be a suction cup, a reducer, or any other structure having similar chemical and mechanical properties. Most preferably, the plastic tubing is polyurethane tubing. Most preferably the chamber seal is made of polyvinylidene fluoride (sold under the name Kynar® by Elf Atochem North America). Diffusion-facilitating means that create a concentration gradient across the membrane are also preferred. Concentration gradients are created, for example, by providing a flow of inert gas across flexible, gas permeable layer **16**, wherein the molecules of the inert gas are too large to pass through flexible, gas permeable layer **16**, while the gas contained in volume **25** passes freely through flexible, gas permeable layer **16**. Those skilled in the art will be able to select the characteristics of flexible, gas permeable layer **16** and gas diffusion accelerators that are appropriate for their selected sample fluid **26**.

Array **17** contained in area **14** on first substrate surface **12** is optionally covered with a water-soluble compound **28**, which protects and seals the biochip prior to use and prevents degradation or other damage to the array. Any water-soluble compound **28** having a melting point of about 30°C to about 60°C, more preferably of about 35°C to about 50°C, and most preferably of about 35°C to about 45°C is advantageously used in filling volume **25** between array **17** and flexible layer **16**. Preferably, the compound is polyethylene glycol, most preferably polyethylene glycol 600. It is a particularly preferred feature of hybridization chamber **10** of the invention that water-soluble compound **28** fills the entirety of the volume **25** and more preferably also fills at least a portion of input port **19**. This prevents formation of air bubbles in volume **25** because compound **28** is first melted, then carefully mixed with the sample fluid **26** within volume **25** using a roller **40** without producing air bubbles in hybridization fluid **26**. The lack of air bubbles in reaction volume **25** enhances efficiency of the biological binding reaction by ensuring that interactions, such as hybridization, between the target analytes and the probes are capable of proceeding without interference from such air bubbles. In addition, it minimizes artifactual signals detected by a scanner **36** or a light pipe **37**.

Ports and holes can be produced in substrate **11** by diamond drilling in glass embodiments of substrate **11** or by stamping or molding in plastic embodiments thereof, or using ceramics formulation technology outlined herein. This facilitates standardization of the hybridization chamber dimensions, for example, by producing substrates where the first and second ports **19** and **20** are produced in a single operation. Both the substrate **11** and the removable cover **21** can be set down as strips or large sheets, and can be rolled to avoid trapping air bubbles. Flexible layer **16** can be applied by vacuum lamination to avoid trapping air, or can be deposited by spinning or flowing liquid plastic over substrate **11** after treatment with adhesive **15** and water-soluble compound **28**, followed by curing the flexible layer in place. Individual hybridization chambers **10** can be produced in stacks using, for example, a diamond saw as shown in Figure 6.

Figure 6 illustrates a preferred arrangement for manufacturing hybridization chamber **10**, wherein alternating layers of flexible layer **16**, adhesive layer **15**, uncut substrate **11**, and removable cover **21** are laid down, and hybridization chambers are produced by cutting the stacked layers, for example, with a diamond saw or any appropriate manufacturing tool. The sealed volumes **25** protect the arrays **17** from debris produced during the cutting process.

Alternative embodiments of the hybridization chamber **10** of the invention encompass a multiplicity of arrays **17** confined in a multiplicity of areas **14** underneath flexible layer **16**, each area comprising an array **17** and being supplied with first port **19** and, optionally, second port **20**. In these embodiments, adhesive layer **15** is deposited on first substrate surface **12** in a pattern that defines each of areas **14**, and flexible layer **16** is applied to adhesive layer **15** to encompass areas **14** on said surface.

In certain embodiments of the invention, hybridization chamber **10** is produced containing array **17** or a multiplicity of arrays **17** as disclosed herein, wherein the chamber is provided ready-to-use by the addition of hybridization fluid **26** comprising one or a multiplicity of target molecules. In alternative embodiments, hybridization chamber **10** is provided without array **17**, and allows for insertion thereof by a user. In these embodiments, at least one edge of flexible layer **16** is not adhered to first substrate surface **12** until array gas diffusion accelerator **17** is inserted.

In the use of the hybridization or reaction chamber **10** of the invention, an amount of a sample fluid **26**, most preferably comprising a biological sample containing a target nucleic acid, is added to the reaction chamber through first port **19**. Before application of the hybridization fluid **26** to the chamber, volume **25** is most preferably heated to a temperature greater than or equal to the melting temperature of water-soluble compound **28**. When melted, hybridization fluid **26** can be added to the chamber and mixed with the water-soluble compound, as shown in Figure 1B. Preferably, water-soluble compound **28** does not affect hybridization in the chamber. More preferably, the amount of compound **28** is chosen such that hybridization efficiency is improved when compound **28** is mixed with sample fluid

26.

In embodiments of the chamber comprising first port **19** but not second port **20**, the hybridization fluid is preferably introduced into the chamber after compound **28** is melted, and then the fluid is cycled into and out of the chamber using, most preferably, a pipette, until fluid **26** and compound **28** are fully mixed, and the hybridization fluid evenly distributed over the surface of array **17**, or mixed into gel pads **22** comprising certain embodiments of said arrays. Alternatively, hybridization fluid **26** is evenly distributed over the surface of array **17**, or mixed into gel pads **22** by physically manipulating flexible layer **16**, as more fully described below. In these embodiments, hybridization fluid **26** is removed after hybridization is completed, as shown in Figure 9, and array **17** is washed by the cycling a sufficient volume of a wash solution **27** into and out of the chamber, most preferably using a pipette.

In embodiments of the chamber comprising both first port **19** and second port **20**, the hybridization fluid is preferably introduced into the chamber after compound **28** is melted, and then the fluid is cycled into and out of the chamber using, most preferably, at least one pipette, until fluid **26** and compound **28** are fully mixed, and the hybridization fluid evenly distributed over the surface of array **17**, or mixed into gel pads **22** comprising certain embodiments of said biochips. Hybridization is then performed by incubating the chamber for a time and at a temperature sufficient for hybridization to be accomplished. Hybridization fluid **26** is removed after hybridization has been completed using outlet port **20**, and the biochip washed by the addition and cycling of a sufficient volume of a wash solution **27** into and out of the chamber, most preferably using a pipette. In these embodiments, the wash solution can also be continuously provided by addition through the input port and removal through the output port. In certain embodiments, the biochip containing the hybridized array is removed from the chamber for development or further manipulations as required. In preferred embodiments, the biochip containing the hybridized array is analyzed *in situ* as described below.

Prior to commencing the reaction, the reaction apparatus **10** is degassed using vacuum source **70**. Preferably a vacuum of between 13 and 27 kPa (100 to 200 torr), more preferably a vacuum of between 13 and 20 kPa (100 to 150 torr), and most preferably a vacuum of about 13 kPa (100 torr) is applied. Preferably the vacuum is applied for between 10 seconds and 2 minutes, more preferably between 10 seconds and 1 minute, most preferably between 10 seconds and 30 seconds. Vacuum source **70** is then detached from flexible, gas permeable layer **16**, and volume **25** is visually inspected for the presence of gas bubbles.

Figure 1B illustrates an advantageous embodiment of hybridization chamber **10** of the invention, further comprising a heating element **33**. Most advantageously, heating element **33** has a heating surface **34** adapted to the shape of hybridization chamber **10** to substantially cover the area **14** under flexible layer **16**. Heating element **33** is any suitable heating means, including but not limited to

resistance heaters, thermoelectric heaters, or microwave absorbing heaters.

The hybridization chamber **10** of the invention also advantageously comprises a thermocouple **35** or other temperature-sensing or measuring element to measure the temperature of hybridization fluid **26** or chamber **10**. These temperature-sensing elements advantageously are coupled with heating element **33** to control hybridization fluid **26** and wash solution **27** temperature, and can be used to calibrate other elements, such as scanning devices **36** as described below that may be sensitive to temperature.

In certain embodiments of the invention, positive hybridization is detected visually, *i.e.*, by the production of a dye or other material that reflects visible light at sites on biochip **18** where hybridization has occurred. In these embodiments, the dye or other material is most preferably produced enzymatically, for example, using a hybridization-specific immunological reagent such as an antibody linked to an enzyme that catalyzes the production of the dye. Visual inspection can be used to detect sites of positive hybridization. More preferably, the biochip containing the hybridized array is scanned using scanner **36** as disclosed more fully below.

Positive hybridization on biochip **18** most preferably is detected by fluorescence using labeled target molecules in a biological sample, or by including intercalating dyes in the hybridization fluid **26** that fluoresce when bound by a hybridized DNA duplex and illuminated by light at a particular wavelength. Suitable intercalating dyes include, but are not limited to, ethidium bromide, Hoechst DAPI, and Alexa Fluor dyes. Suitable fluorescence labels include, but are not limited to, fluorescein, rhodamine, propidium iodide, and Cy3 and Cy5 (Amersham), that can be incorporated into target molecules, for example, *in vitro* amplified fragments using labeled oligonucleotide primers.

Figures 10A-10C illustrate an embodiment of the invention comprising a scanner **36**, which is advantageously positioned over (or beneath) flexible layer **16** and moves from one end of area **14** to the opposite end, sequentially illuminating area **14** and array **17** positioned thereupon. Prior to analysis of the hybridized array, all fluid is removed from volume **25** such that flexible layer **16** is in contact with array **17**. Scanner **36** then excites the fluorescent dye, preferably with short wavelength light, most preferably light with a wavelength between 250 nm and 600 nm. Scanner **36** then collects the emitted light from a specific area. The amount of light emitted is then used to determine the amount of fluorescent dye present in the area, and hence the amount of labeled target.

Particular embodiments of scanners and scanning devices **36** are shown in Figures 11A through 11E. It is a particularly advantageous feature of hybridization chamber **10** that flexible layer **16** is translucent to suitable wavelengths of light, including light in the ultraviolet and visible portion of the spectrum. An additional advantageous feature of hybridization chamber **10** is that flexible layer **16**, which is very thin,

is immediately adjacent to and in contact with biochip **18** after hybridization fluid **26** or wash fluid **27** is removed from the chamber. This combination of features reduces or eliminates free surface reflections, internal reflection of illumination from the scanner, and dispersion or scattering of illuminating light, thereby optimizing the amount of incident light that illuminates array **17**. This arrangement is also more economical than in existing apparatus as it minimizes the need for highly polished, low scattering surfaces or complex or expensive lenses, and eliminates problems associated with focus and depth-of-field in more complex optical detectors.

In other embodiments, a light pipe **37** contacts the surface of flexible layer **16** that is immediately adjacent to and in contact with the surface of array **17**, as shown in Figure 11B. In these embodiments, both illuminating and emitted light are conveyed and collected by light pipe **37**. The pipe is designed to be slightly flexible so as to adapt to the contoured surface of flexible layer **16**. Light pipe **37** contacts flexible layer **16** that contacts array **17**, thereby permitting contacts free of surface reflections even under circumstances where array **17** or light pipe **37** has localized variations in height. Advantageously, light pipe **37** has a larger surface area than array **17**, so that the maximum amount of illuminating light is delivered to array **17**, and the maximum amount of emitted light from array **17** is collected by light pipe **37**. A further advantage of light pipe **37** is that it enables detection of bubbles formed in hybridization fluid **26** or wash buffer **27**, which detection can be used as a signal for roller **40** to address flexible layer **16** to remove such bubbles. Removing bubbles in hybridization fluid **26** or wash buffer **27** reduces the frequency of non-specific binding and artifactual signals detected by scanner **36**.

In additional embodiments of the invention, the area **14** defined by adhesive layer **15** further comprises a reflective layer **38** that substantially covers the entirety of the area **14** and is positioned between array **17** and the first substrate surface **12**. In preferred embodiments, reflective layer **38** comprises aluminum, gold, silver, or platinum. In these embodiments, the amount of the light signal reflected or transmitted to the light-detecting portion of scanner **36** is increased up to four-fold. In further advantageous embodiments of the invention, reflective layer **38** is a metal film resistor or an RF induction heater. In these embodiments, reflective **38** layer can heat the slide without requiring additional heating elements **33**. This is a particularly desirable feature in hand-held embodiments of the hybridization chamber **10** of the invention.

If required, a passivation later **39** can be applied on top of reflective layer **38**. Preferably, passivation layer **39** is a layer of parylene a few microns thick that is applied by evaporation. The amount of illumination required, and hence the amount of power needed to operate scanner **36** is reduced in these embodiments, which are particularly suited to battery-operated embodiments such as hand-held devices to improve useful battery life. Furthermore, passivation layer **39** reduces artifactual signals in the light emission data by obscuring objects that it covers.

Hybridization chamber **10** is preferably supplied with a roller **40** in removable contact with flexible layer **16** and that can be moved longitudinally across areas **14** on first substrate surface **12**. In preferred embodiments, the surface of roller **40** comprises a textured pattern **41**, most preferably a spiral pattern, that permits the roller to efficiently mix hybridization fluid and wash solution across area **14** and array **17**. The roller can move longitudinally across the surface of the chamber for mixing sample fluid and wash solutions as required. One advantageous arrangement of roller **40** (again, preferably a patterned roller) and hybridization chamber **10** is shown in Figure 11E. As shown in the Figure, roller **40** can be advantageously connected to a movable arm **42** that can be positioned to place roller **40** in contact with flexible layer **16** when in a first position, and can be moved to a second position in which roller **40** is not in contact with flexible layer **16**. Most preferably, movable arm **42** has a pivot point **44** and movement about said pivot point is preferably controlled by a solenoid. In addition to movement of roller **40** in contact with and away from hybridization chamber **10**, either roller **40** or hybridization chamber **10**, or both, are movable in a longitudinal direction to enable roller **40** to mix hybridization fluid **26** or wash solution **27** inside volume **25** bounded by flexible layer **16**, adhesive layer **15**, and first substrate surface **12** in area **14** containing array **17**. In embodiments comprising a multiplicity of areas **14** containing a multiplicity of arrays **17**, roller **40** is positioned to move longitudinally across each of the multiplicity of areas **14** to mix hybridization fluid **26** or wash solution **27** in each of the volumes **25** containing arrays **17**.

In additional embodiments, a sample preparation chip **45**, comprising a port **46**, as shown in Figures 12A through 12C, can be attached to hybridization chamber **10**. Most preferably, port **46** in sample preparation chip **45** is aligned with first port **19** in hybridization chamber **10** to permit efficient transfer of sample to volume **25**. Additional fiducial references can be used to accurately align hybridization chamber **10** and sample preparation chip **45**. Since access to first port **19** is through second substrate surface **13**, the array can be scanned without interference from the attached sample preparation chip. In alternative embodiments of the invention, sample preparation chip **45** may be bound to second substrate surface **13** (Figure 12B) or formed as an integral part of substrate **11** (Figure 12C).

A preferred embodiment of hybridization chamber **10** of the invention is a hand-held embodiment as shown in Figures 10A-10C, further comprising a scanner **36**. In these embodiments, hand-held device **47** comprises a base **48**, a lid **49** and a carriage **50** embodying roller **40**, scanner **36**, heating element **33** and thermocouple **35**. Carriage **50** is illustrated in Figure 11A. Device **47** comprises a compartment **51** for positioning hybridization chamber **10** in proximity to carriage **50**. Carriage **50** is provided with moving means for moving roller **40**, scanner **36** and heating element **33** relative to hybridization chamber **10** as required for operation as described above. Carriage **50** and lid **49** are arranged to permit a user to introduce and remove hybridization fluid **26** and wash solution **27** into the chamber through first port **19** and second port **20** as required. Alternatively, device **47** further comprises fluidic connections **52** to each of the first and second ports to provide for sample

introduction and array washing after hybridization of the sample thereto. Device **47** is most preferably operated by battery, although AC adapters are also advantageously encompassed by the description of the device herein. In further preferred embodiments, lid **49** further comprises a display **56** for displaying the results of the analysis.

With respect to the methods of using the devices, there are a wide variety of methods that can be used. If required, the target sequence is prepared using known techniques. For example, the sample may be treated to lyse the cells, using known lysis buffers, sonication, electroporation, etc., with purification and amplification as outlined below occurring as needed, as will be appreciated by those in the art. In addition, the reactions outlined herein may be accomplished in a variety of ways, as will be appreciated by those in the art. Components of the reaction may be added simultaneously, or sequentially, in any order, with preferred embodiments outlined below. In addition, the reaction may include a variety of other reagents which may be included in the assays. These include reagents like salts, buffers, neutral proteins, e.g. albumin, detergents, etc., which may be used to facilitate optimal hybridization and detection, and/or reduce non-specific or background interactions. Also reagents that otherwise improve the efficiency of the assay, such as protease inhibitors, nuclease inhibitors, anti-microbial agents, etc., may be used, depending on the sample preparation methods and purity of the target.

In addition, in most embodiments, double stranded target nucleic acids are denatured to render them single stranded so as to permit hybridization of the primers and other probes of the invention. A preferred embodiment utilizes a thermal step, generally by raising the temperature of the reaction to about 95°C, although pH changes and other techniques may also be used.

As outlined herein, the invention provides a number of capture probes that will hybridize to some portion, i.e. a domain, of the target sequence. Probes of the present invention are designed to be complementary to a target sequence (either the target sequence of the sample or to other probe sequences, for example for use in sandwich assays known in the art) such that hybridization of the target sequence and the probes of the present invention occurs. As outlined below, this complementarity need not be perfect; there may be any number of base pair mismatches which will interfere with hybridization between the target sequence and the single stranded nucleic acids of the present invention. However, if the number of mutations is so great that no hybridization can occur under even the least stringent of hybridization conditions, the sequence is not a complementary target sequence. Thus, by "substantially complementary" herein is meant that the probes are sufficiently complementary to the target sequences to hybridize under normal reaction conditions.

A variety of hybridization conditions may be used in the present invention, including high, moderate and low stringency conditions; see for example Maniatis et al., Molecular Cloning: A Laboratory Manual, 2d Edition, 1989, and Short Protocols in Molecular Biology, ed. Ausubel, et al, hereby

incorporated by reference. Stringent conditions are sequence-dependent and will be different in different circumstances. Longer sequences hybridize specifically at higher temperatures. An extensive guide to the hybridization of nucleic acids is found in Tijssen, Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Acid Probes, "Overview of principles of hybridization and the strategy of nucleic acid assays" (1993). Generally, stringent conditions are selected to be about 5-10°C lower than the thermal melting point (Tm) for the specific sequence at a defined ionic strength and pH. The Tm is the temperature (under defined ionic strength, pH and nucleic acid concentration) at which 50% of the probes complementary to the target hybridize to the target sequence at equilibrium (as the target sequences are present in excess, at Tm, 50% of the probes are occupied at equilibrium). Stringent conditions will be those in which the salt concentration is less than about 1.0 M sodium ion, typically about 0.01 to 1.0 M sodium ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes (e.g. 10 to 50 nucleotides) and at least about 60°C for long probes (e.g. greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of helix destabilizing agents such as formamide. The hybridization conditions may also vary when a non-ionic backbone, i.e. PNA is used, as is known in the art. In addition, cross-linking agents may be added after target binding to cross-link, i.e. covalently attach, the two strands of the hybridization complex.

Thus, the assays are generally run under stringency conditions which allows formation of the hybridization complex only in the presence of target. Stringency can be controlled by altering a step parameter that is a thermodynamic variable, including, but not limited to, temperature, formamide concentration, salt concentration, chaotropic salt concentration, pH, organic solvent concentration, etc.

These parameters may also be used to control non-specific binding, as is generally outlined in U.S. Patent No. 5,681,697. Thus it may be desirable to perform certain steps at higher stringency conditions to reduce non-specific binding.

As described herein, there are a number of possible detection techniques that can be utilized in the present invention. In a preferred embodiment, as outlined herein, optical label techniques are used. In these embodiments, a label such as an optical dye (e.g. a fluorochrome) is added to the assay complex comprising the target analyte and the capture binding ligand. In some embodiments, for example in the case of nucleic acids, the label can be added to the target, for example by incorporation during an amplification reaction such as PCR. For example, the fluorochromes or other labels such as biotin can be added to the PCR primers or to the dNTPs for enzymatic incorporation. Alternatively, intercalators can be used as described above.

Alternatively, preferred embodiments allow the use of electrical detection methods such as those outlined in U.S.S.N.s 09/458,553; 09/458,501; 09/572,187; 09/495,992; 09/344,217; WO00/31148; 09/439,889; 09/438,209; 09/344,620; 09/478,727; PCTUS00/17422; WO 98/20162; WO 98/12430;

WO 98/57158; WO 99/57317; WO 99/67425; PCT 00/19889; and WO 99/57319, all of which are expressly incorporated by reference in their entirety. These embodiments utilize arrays of microelectrodes on the substrate.

The Examples that follow are illustrative of specific embodiments of the invention and various uses thereof. They are set forth for explanatory purposes only, and are not to be taken as limiting the invention. All references cited herein are expressly incorporated by reference in their entirety.

EXAMPLE 1

ASSEMBLY OF A HYBRIDIZATION CHAMBER

The process of assembling a chamber according to the present invention is illustrated in Figure 13.

A die cutter was used to cut four ellipsoidal holes in a layer of 502FL ultra-clean laminating adhesive film (3M). A similar pattern was punched into an Avery laser label 5663 for use as a frame and label layer. Meanwhile, a sheet of polyvinylidene chloride film was stretched over a stainless steel frame and annealed for 30 minutes at 100°C. The Avery label was applied to one side of the polyvinylidine chloride film by vacuum laminating the label in a vacuum lamination press. A vacuum of 15 psi was applied for 30 seconds, and mechanical pressure of 15 psi was maintained for 1 minute after release of the vacuum. The adhesive was then applied to the opposite side of the polyvinylidene chloride film using the same process as for the label.

The adhesive coated film was then applied to a glass slide that had previously been prepared. The arrays of oligonucleotide probes and gel pads were positioned on the glass slide using standard methods. Ports were drilled into the slide using a diamond drill. A vacuum lamination press was used to affix the polyvinylidene chloride film to the slide. A vacuum of 15 psi was maintained for 1 minute, and then mechanical pressure of 15 psi was maintained for an additional minute.

The individual chambers were then filled with polyethylene glycol 600 using a 10 mL pipette tip. A layer of 3M 7350 polyester tape was then applied to the slide to seal off the ports.

EXAMPLE 2

ASSEMBLY OF A TOP-LOADING HYBRIDIZATION CHAMBER

A die cutter was used to cut four ellipsoidal holes in a layer of 502FL ultra-clean laminating adhesive film (3M). A similar pattern was punched into an Avery laser label 5663 for use as a frame and label layer. Meanwhile, a sheet of polyvinylidene chloride film was stretched over a stainless steel frame and annealed for 30 minutes at 100°C. The Avery label was applied to one side of the polyvinylidine chloride film by vacuum laminating the label in a vacuum lamination press. A vacuum of 15 psi was applied for 30 seconds, and mechanical pressure of 15 psi was maintained for 1 minute after release of the vacuum. The adhesive was then applied to the opposite side of the polyvinylidene chloride film using the same process as for the label.

The adhesive coated film was then applied to a glass slide that had previously been prepared. The arrays of oligonucleotide probes and gel pads were positioned on the glass slide using standard methods. A vacuum lamination press was used to affix the polyvinylidene chloride film to the slide. A vacuum of 15 psi was maintained for 1 minute, and then mechanical pressure of 15 psi was maintained for an additional minute.

The individual chambers were then filled with polyethylene glycol 600 using a 10 mL pipette tip. A layer of 3M 7350 polyester tape was then applied to the slide to seal off the ports.

EXAMPLE 3

REMOVING GAS BUBBLES FROM A REACTION CHAMBER

The process of assembling a chamber according to the present invention is illustrated in Figure 14.

A four reaction-chamber apparatus is manufactured using a layer of 0.2 µm porous Teflon unsupported membrane as the flexible, gas permeable layer, following the procedure provided in U.S. Application Serial No. 09/464,490, incorporated by reference herein. Each reaction chamber is filled with 75 µL of a sample fluid containing biological target molecules by injection through a 300 µL pipette tip (VWR Part No. 53510-084) using a 200 µL pipettor (Rainin P-200). Bubbles are visually detectable in the chambers after injection.

A reaction chamber is isolated by applying a Cole-Parmer Kynar 1/4" x 5/8" barbed reducer (Part No. 31513-31) directly to the frame layer and forming a seal around the chamber. A "house" vacuum source is connected to the reducer by a length of polyurethane tubing. A vacuum of 200 torr is applied for two minutes. Visual inspection of the chamber following application of the vacuum shows no gas bubbles remaining in the chamber.

The reaction apparatus is maintained at 25°C and atmospheric pressure for 8 hours until the reaction proceeds to completion. No appreciable evaporation of water from the chamber is observed.

It should be understood that the foregoing disclosure emphasizes certain specific embodiments of the invention and that all modifications or alternatives equivalent thereto are within the spirit and scope of the invention as set forth in the appended claims.

CLAIMS

We claim:

1. An apparatus for performing biological reactions comprising:
   a) a substrate having a first surface and a second surface;
   b) an array of biomolecules positioned on said first surface;
   c) a porous flexible layer affixed to said first surface by an adhesive layer to create a reaction volume;
   d) a gas diffusion accelerator; and
   e) a first port extended from said second surface to said reaction volume of said first surface.

2. An apparatus according to claim 1 wherein said biomolecules are nucleic acids.

3. An apparatus according to claim 1 or 2 wherein said substrate is glass, silicon, ceramic or plastic.

4. An apparatus according to claim 1, 2 or 3 wherein said biomolecules are nucleic acids.

5. An apparatus according to claim 1, 2, 3 or 4 wherein said biomolecules are attached to said substrate using a gel pad.

6. An apparatus according to claim 1, 2, 3, 4 or 5 wherein said reaction volume comprises a water-soluble compound that is a solid at a first temperature and a liquid at a second higher temperature.

7. An apparatus according to claim 1, 2, 3, 4, 5 or 6 wherein said porous flexible layer is porous Teflon™.

8. An apparatus according to claim 1, 2, 3, 4, 5, 6 or 7 wherein said gas diffusion accelerator comprises a vacuum source affixed to said porous flexible layer.

9. An apparatus according to claim 8 wherein said vacuum source comprises:
   a) a vacuum pump; and
   b) a chamber seal affixed to said reaction volume.

10. A method of detecting the presence of a target analyte in a sample comprising:
   a) contacting said sample with an apparatus comprising:
      i) a substrate having a first surface and a second surface;
      ii) an array of biomolecules positioned on said first surface;
      iii) a porous flexible layer affixed to said first surface by an adhesive

layer to create a reaction volume;

iv) a gas diffusion accelerator; and

v) a first port extended from said second surface to said reaction volume of said first surface;

under conditions whereby said target analyte will bind to at least one of said biomolecules;

b) utilizing said gas diffusion accelerator to remove gas bubbles; and

c) detecting the presence of said target analyte.

11. A method according to claim 10 wherein said biomolecules are nucleic acids.

FIG. 1A

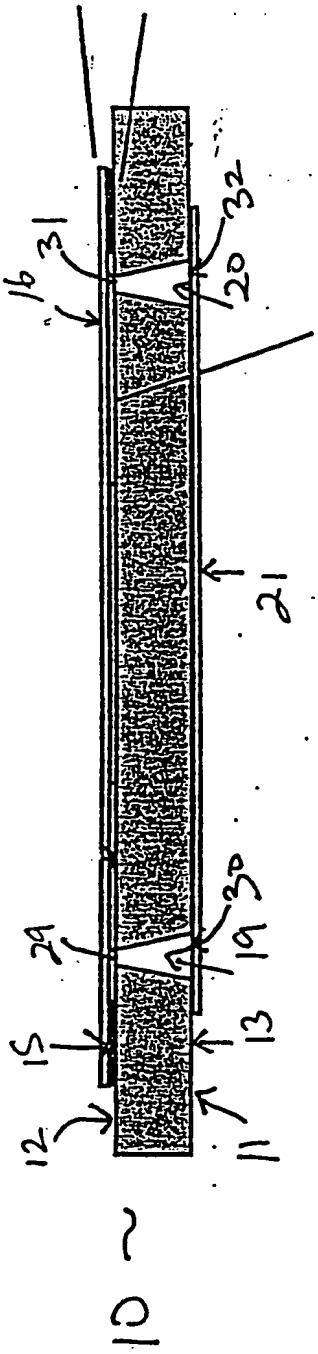


FIG 1B

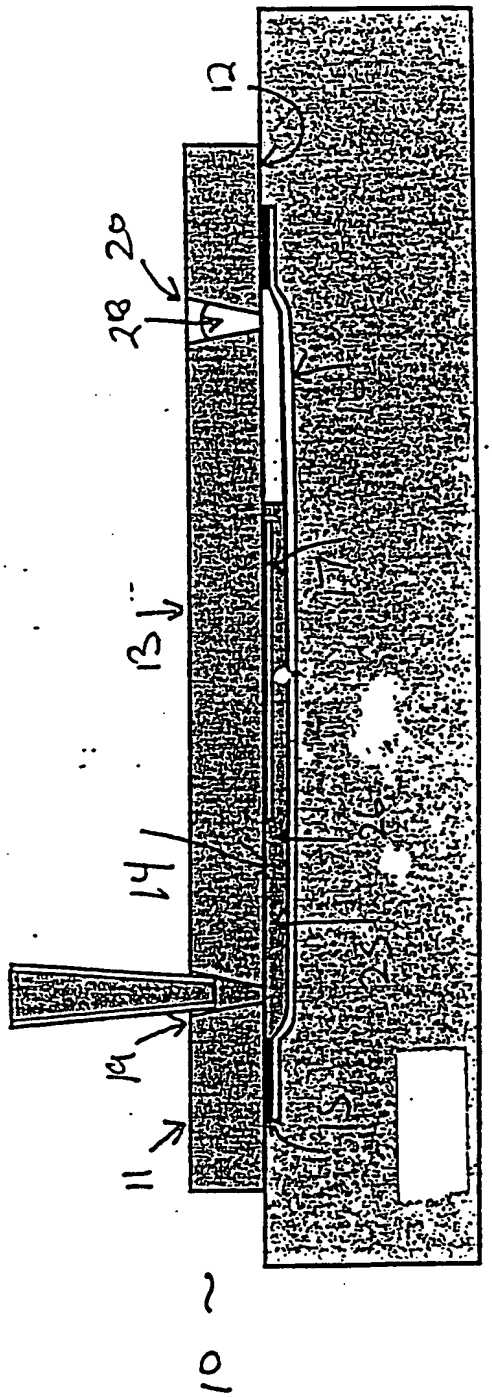


FIG 1C

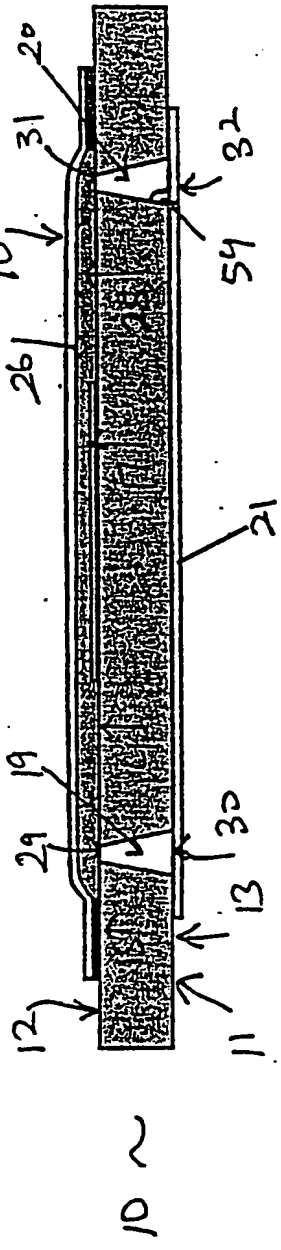
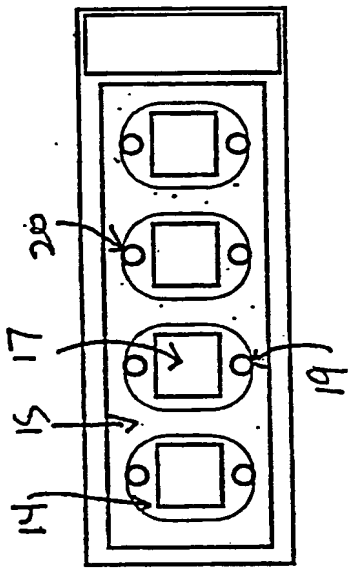


FIG 1D



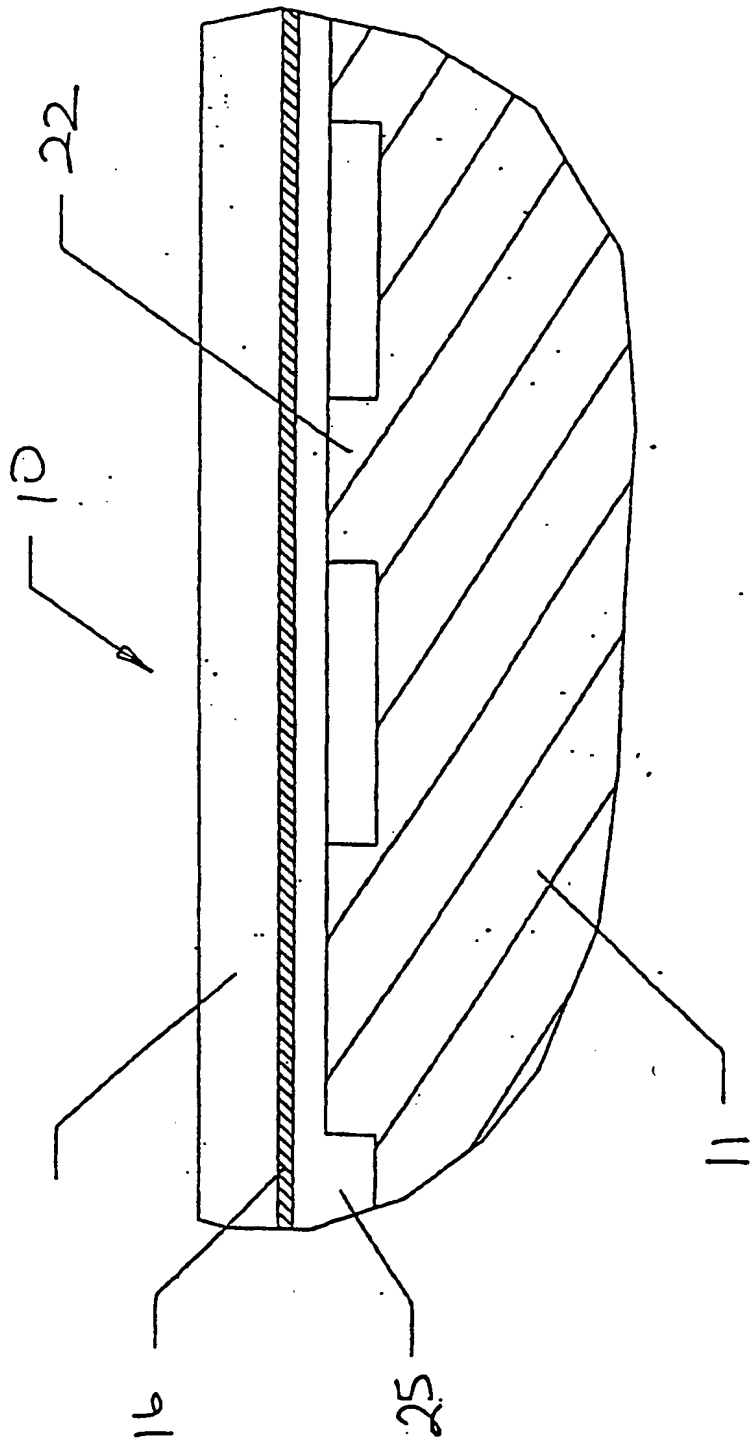


FIGURE 2

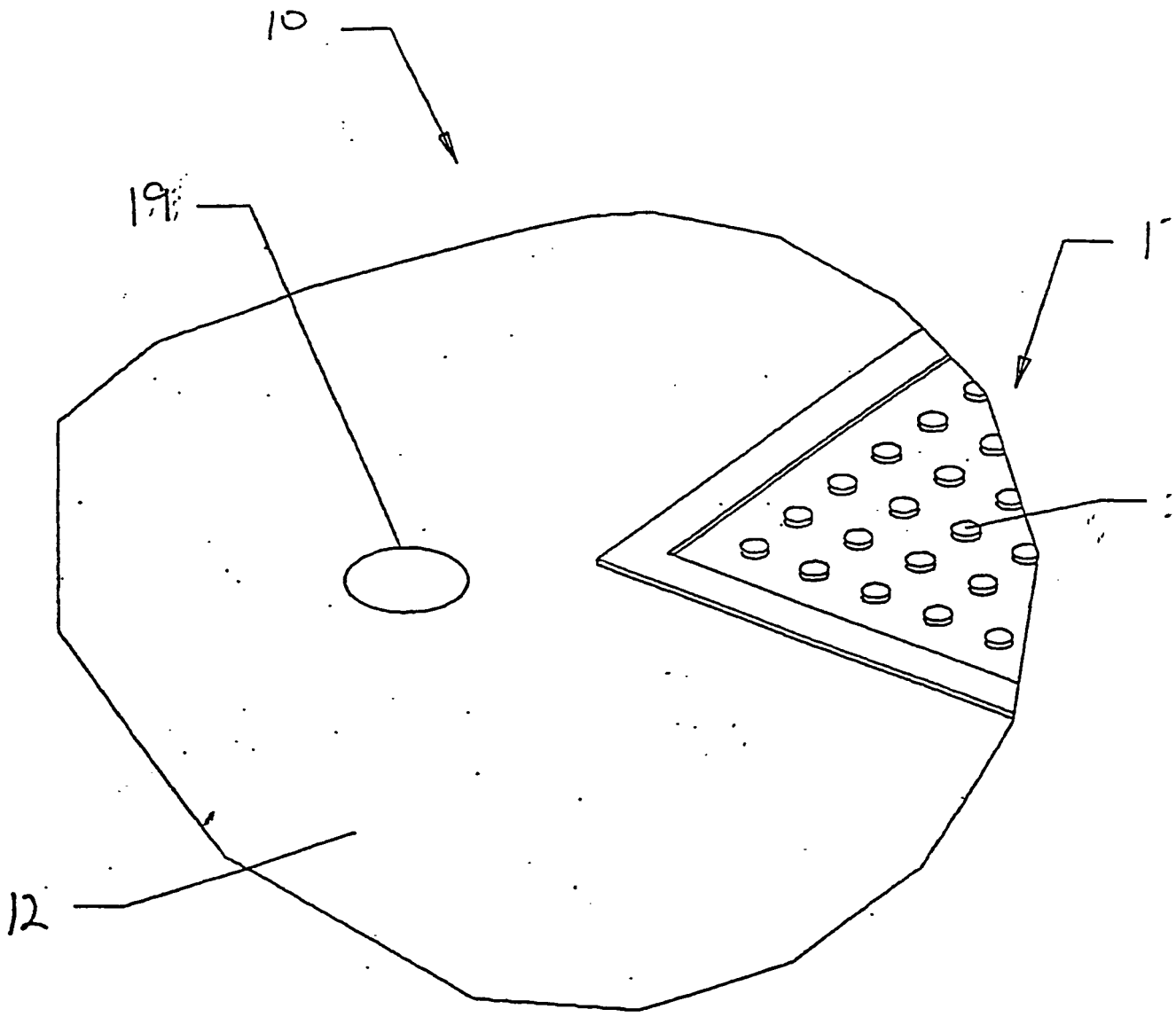


FIGURE 3

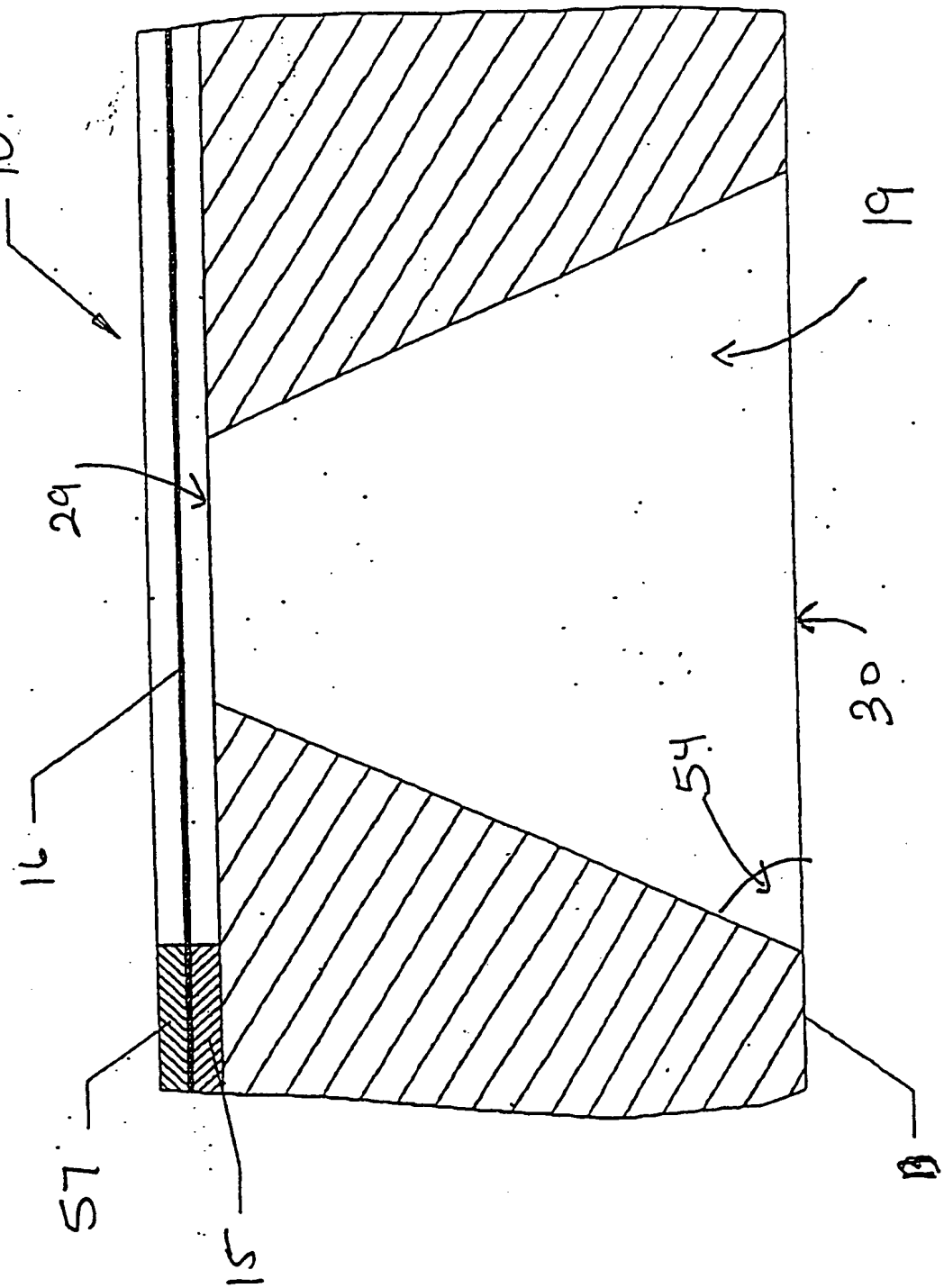


FIGURE 4

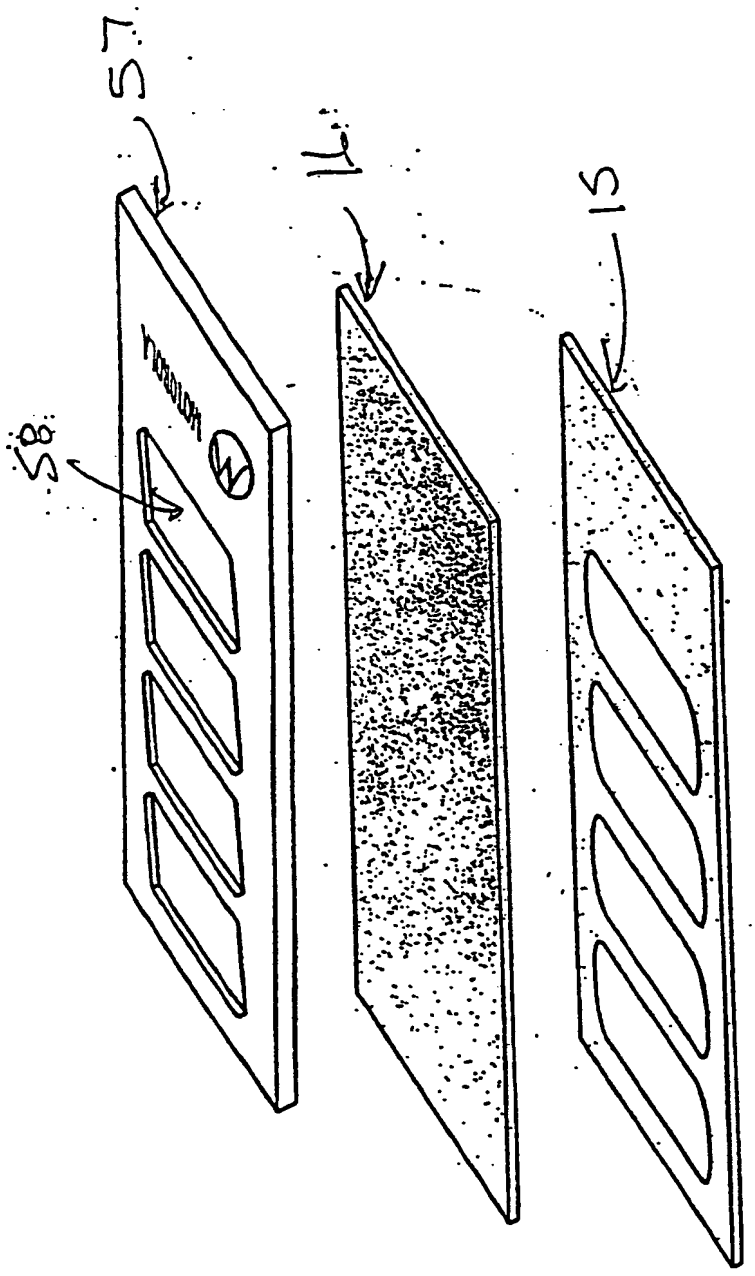


FIG. 5

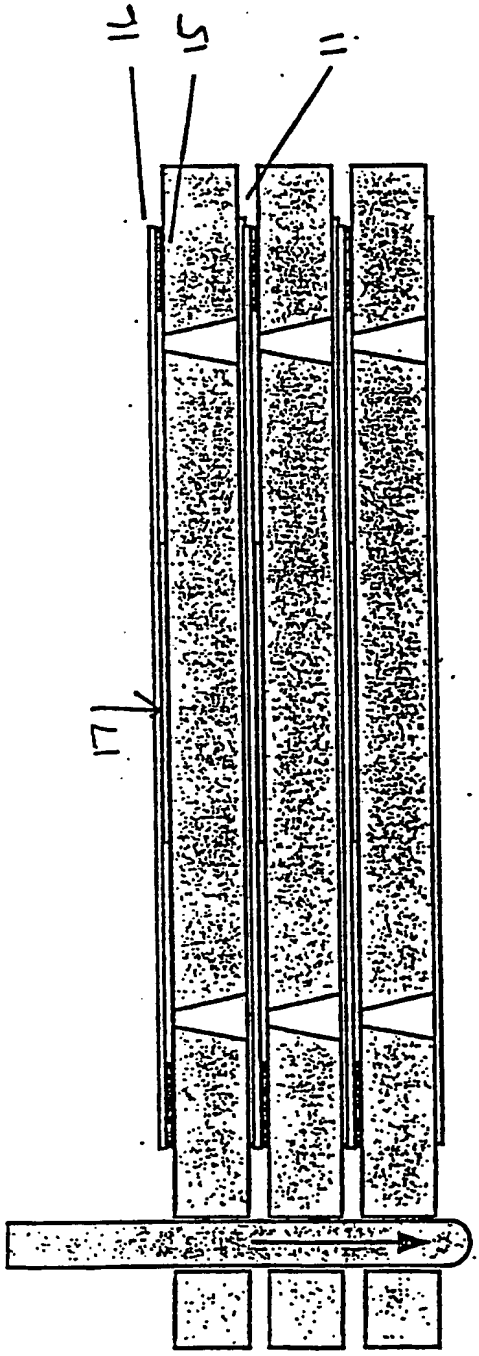


FIG. 6

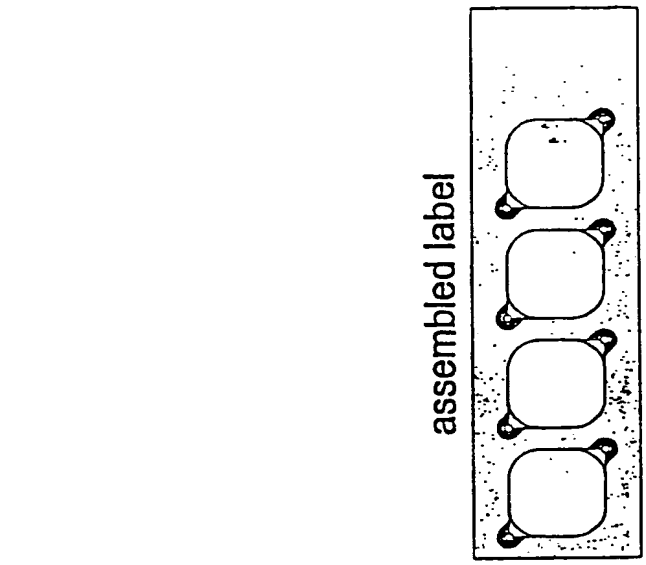


FIG. 7E

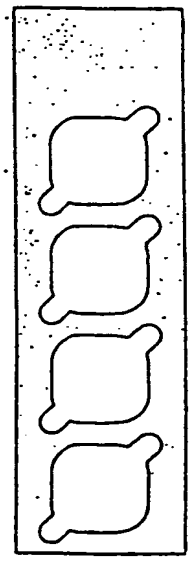


FIG. 7A

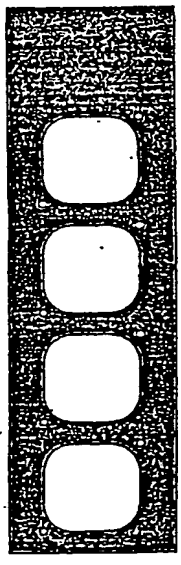


FIG. 7C

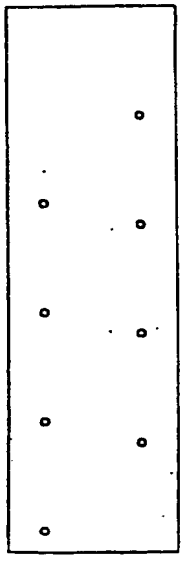


FIG. 7B

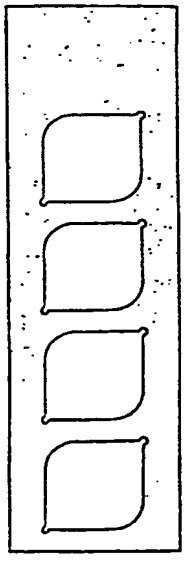


FIG. 7A

FIG. 8A

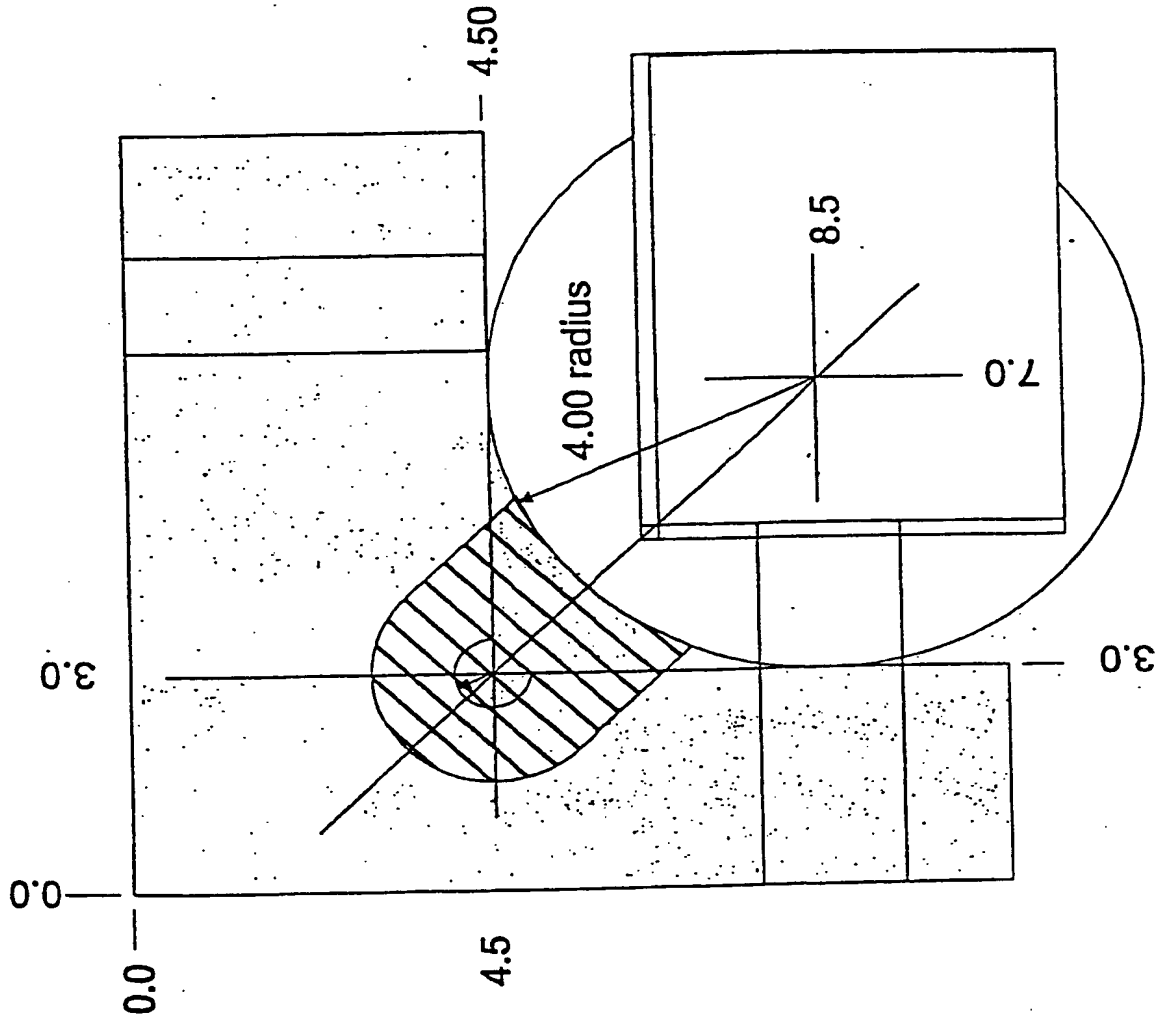
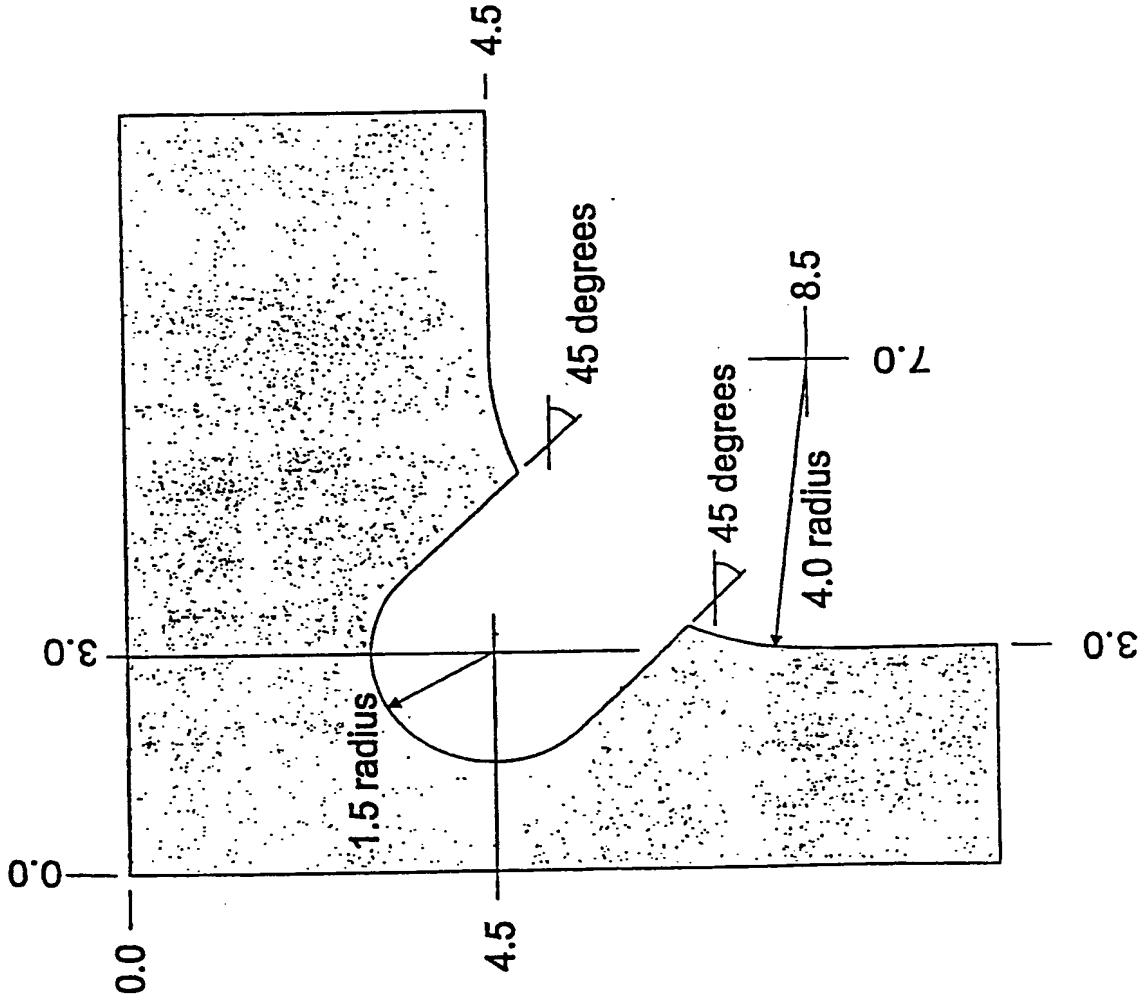


FIG 8B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8.5
7.0
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4.0 radius
3.0
3.0
1.5 radius
0.0
0.0
4.5

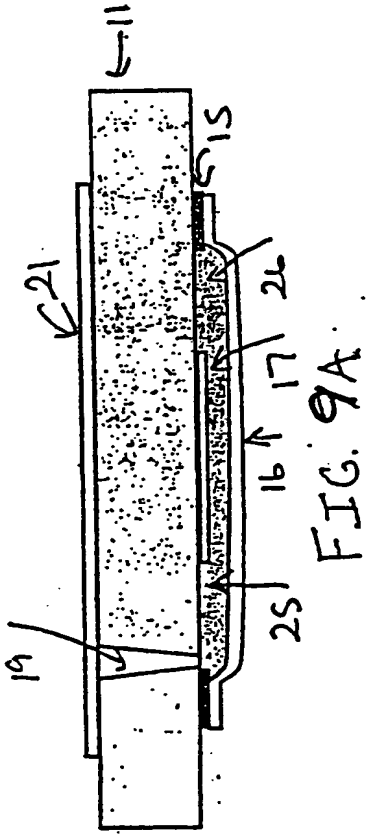


FIG. 9A

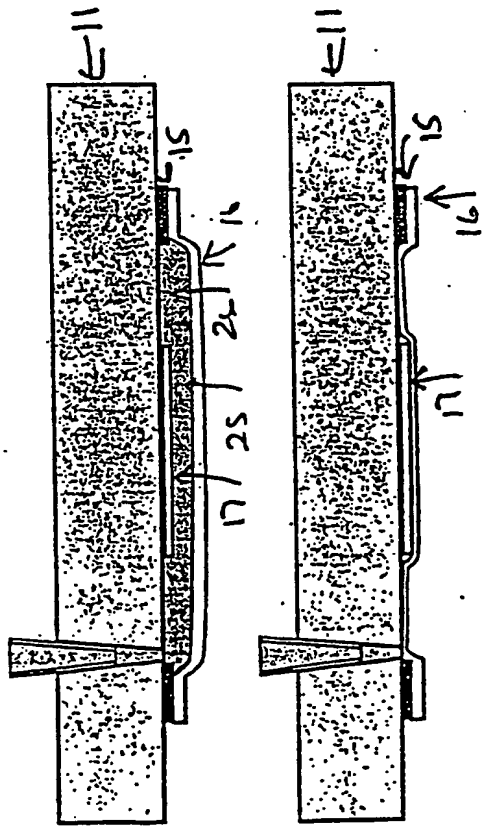


FIG. 9B

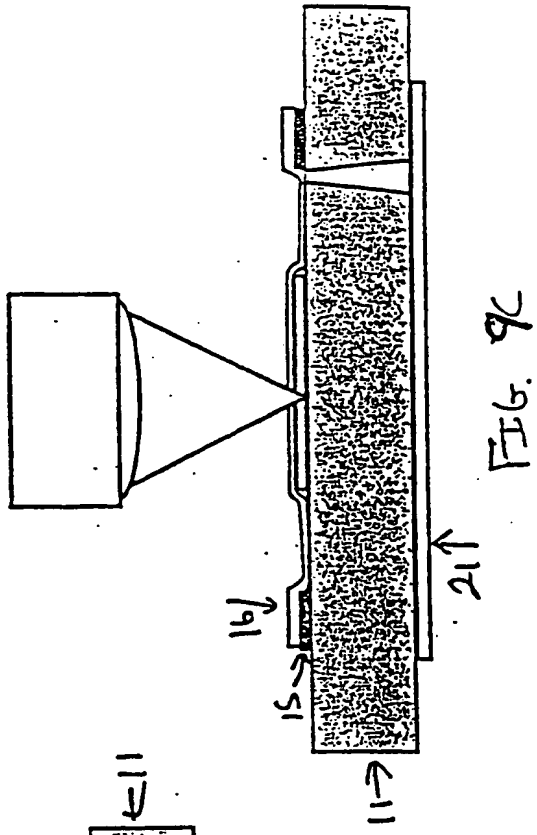


FIG. 9C

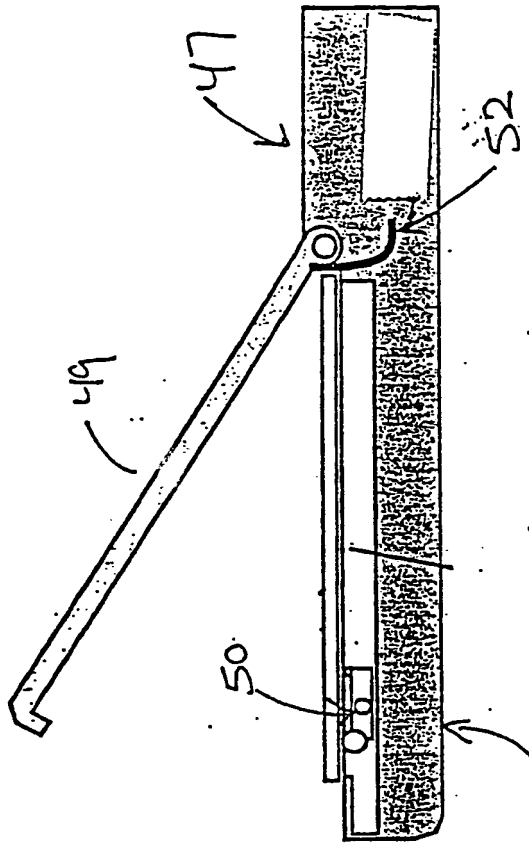


FIG. 10A

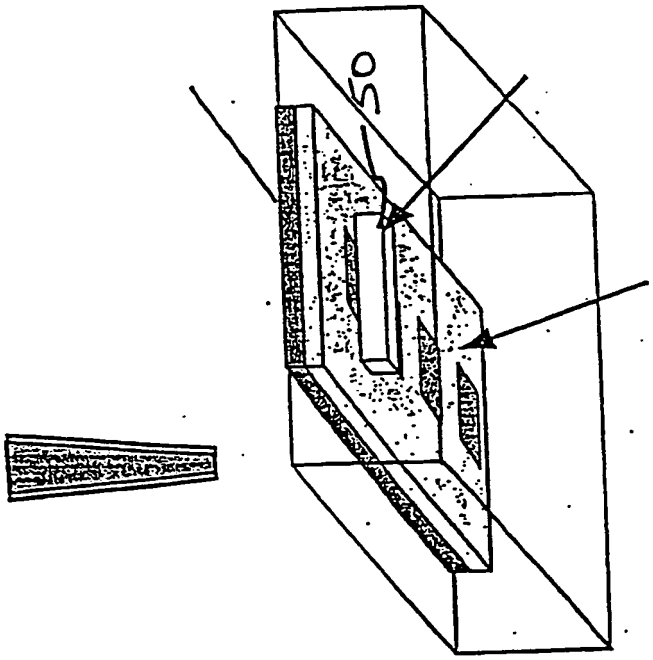
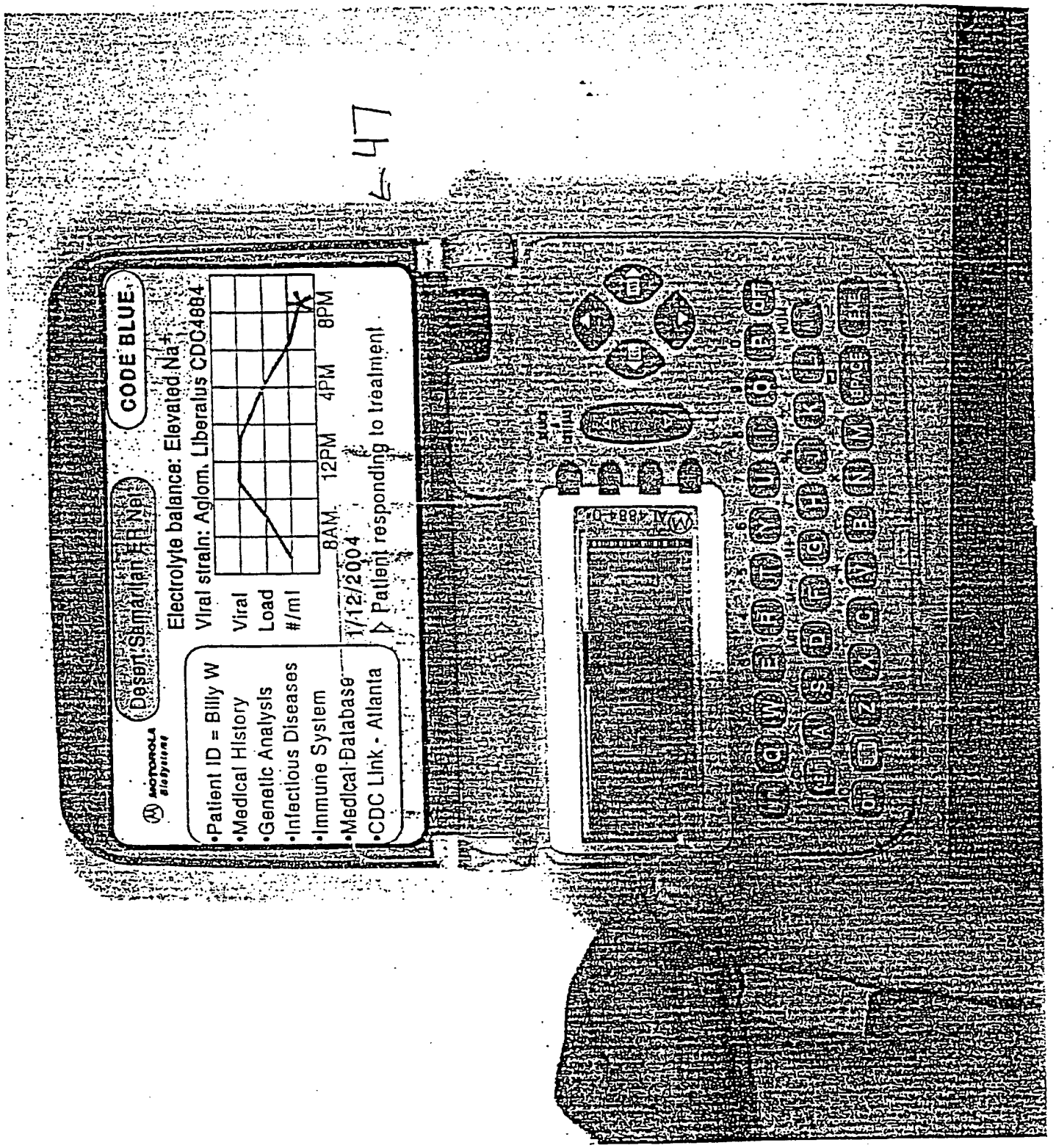


FIG. 10B


CODE BLUE
Desert Samaritan ER Net
Electrolyte balance: Elevated Na+
Viral strain: Aglom. Liberalus CDC4884
Viral Load #/ml
8AM 12PM 4PM 8PM
1/12/2004
Patient responding to treatment
•Patient ID = Billy W
•Medical History
•Genetic Analysis
•Infectious Diseases
•Immune System
•Medical Database
•CDC Link - Atlanta
47

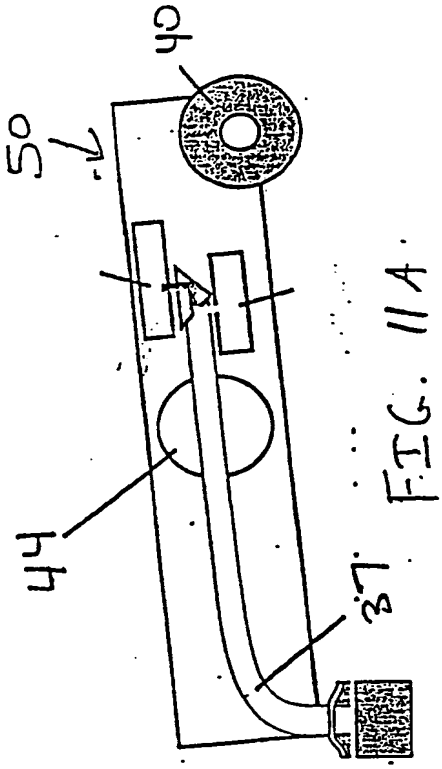


FIG. 11A

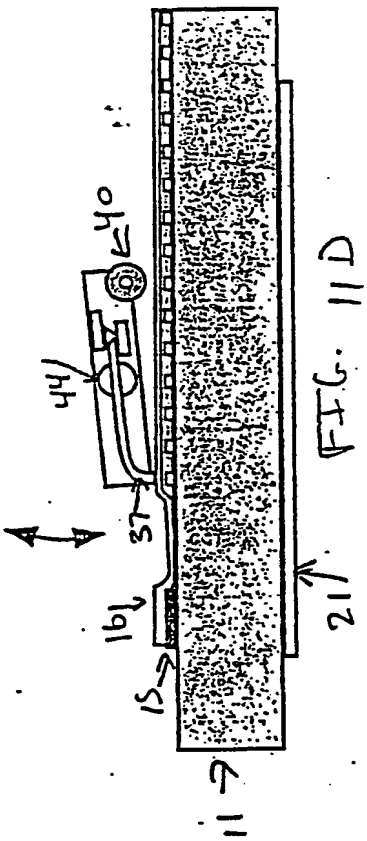


FIG. 11D

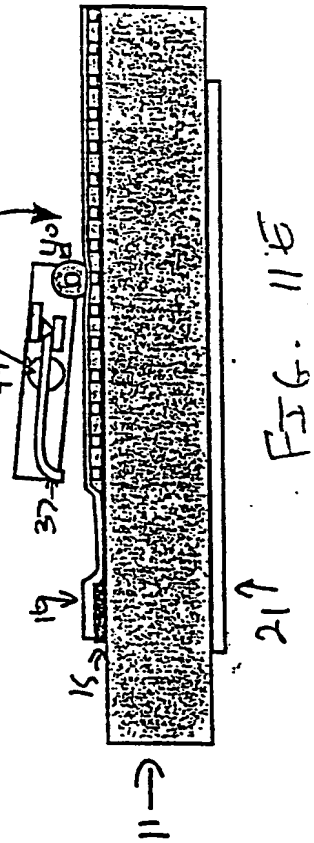


FIG. 11E

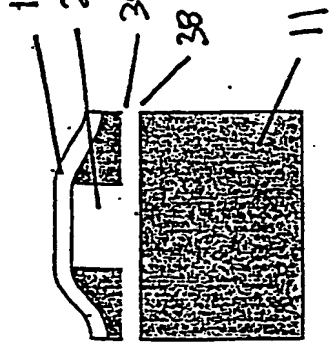


FIG 11C

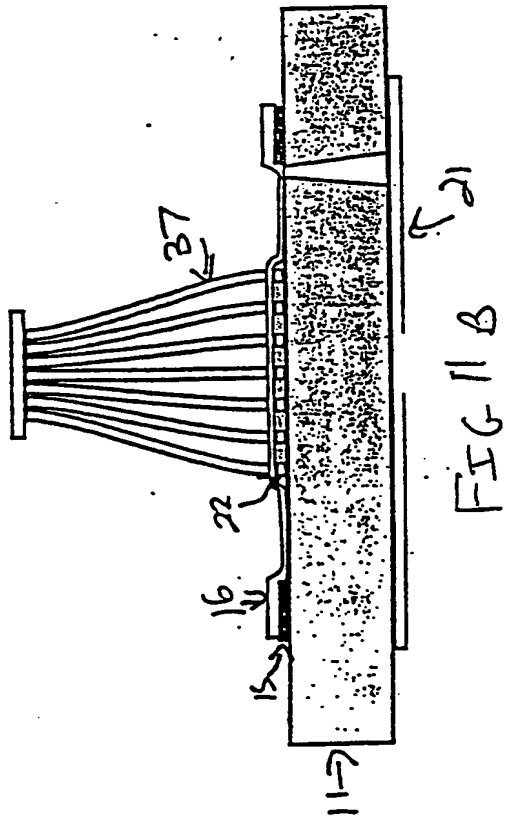


FIG 11B

FIG 12A

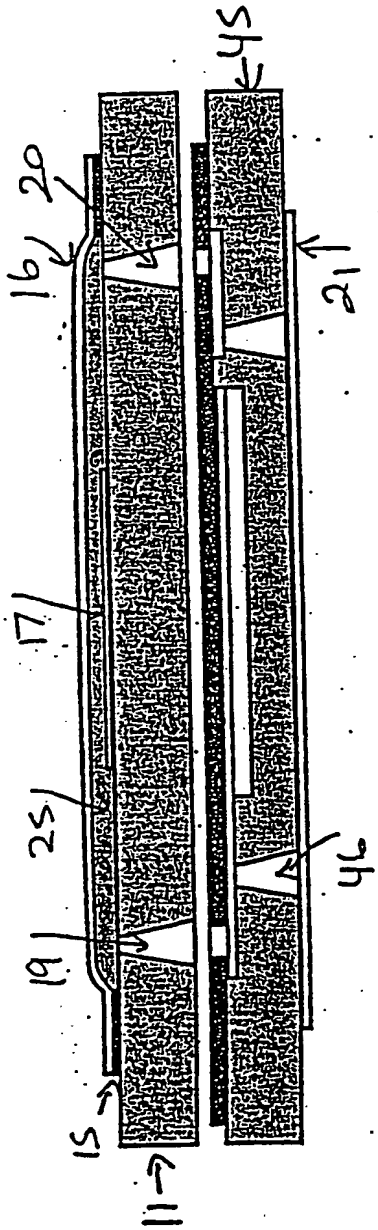


FIG 12B

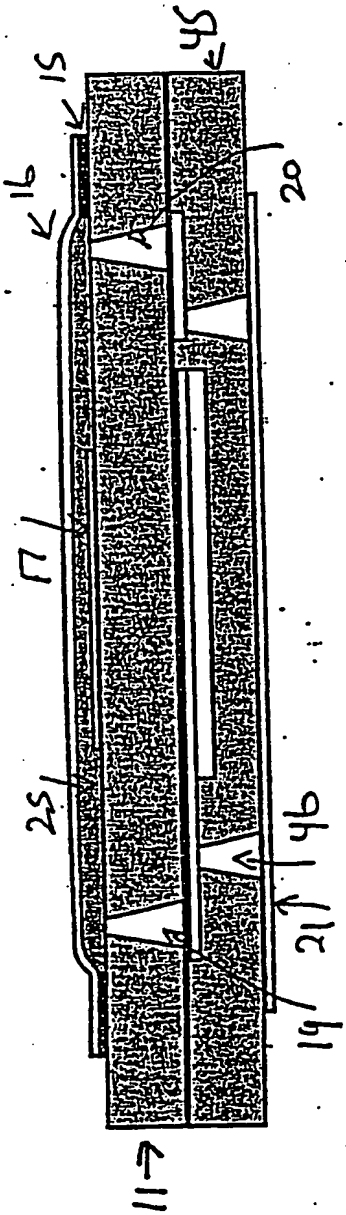
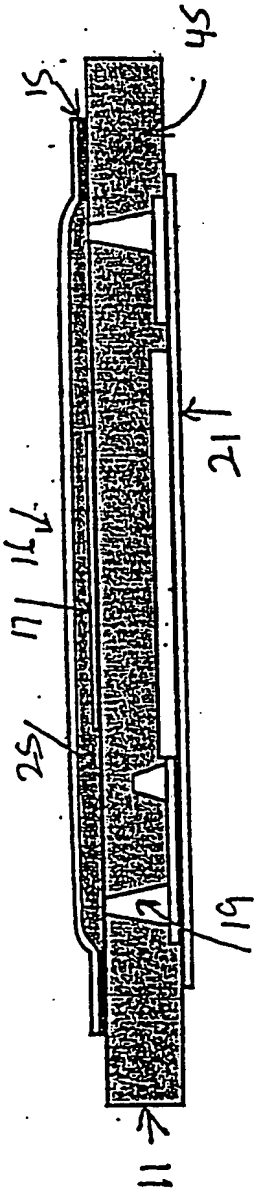


FIG 12C



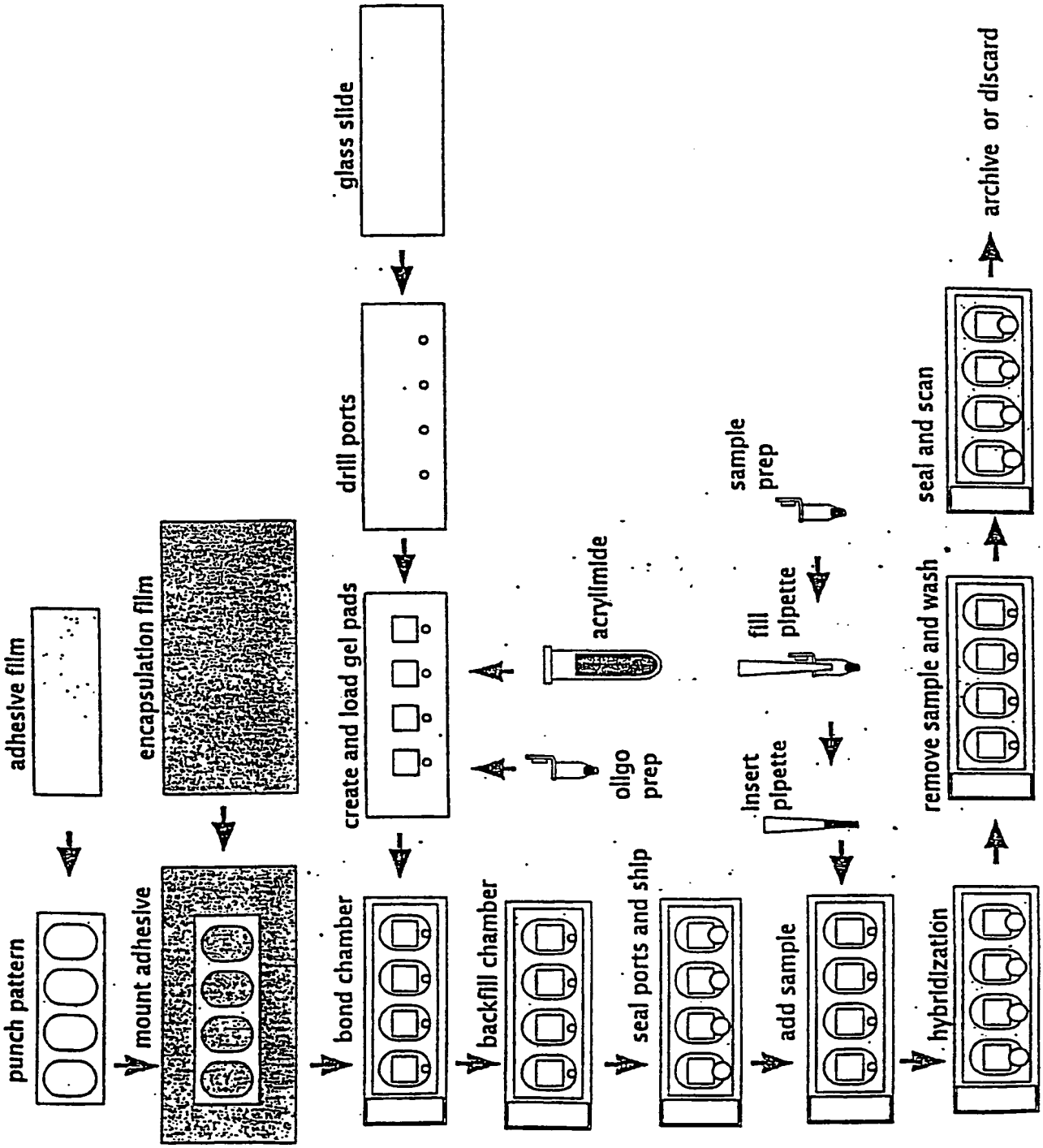


FIGURE 13

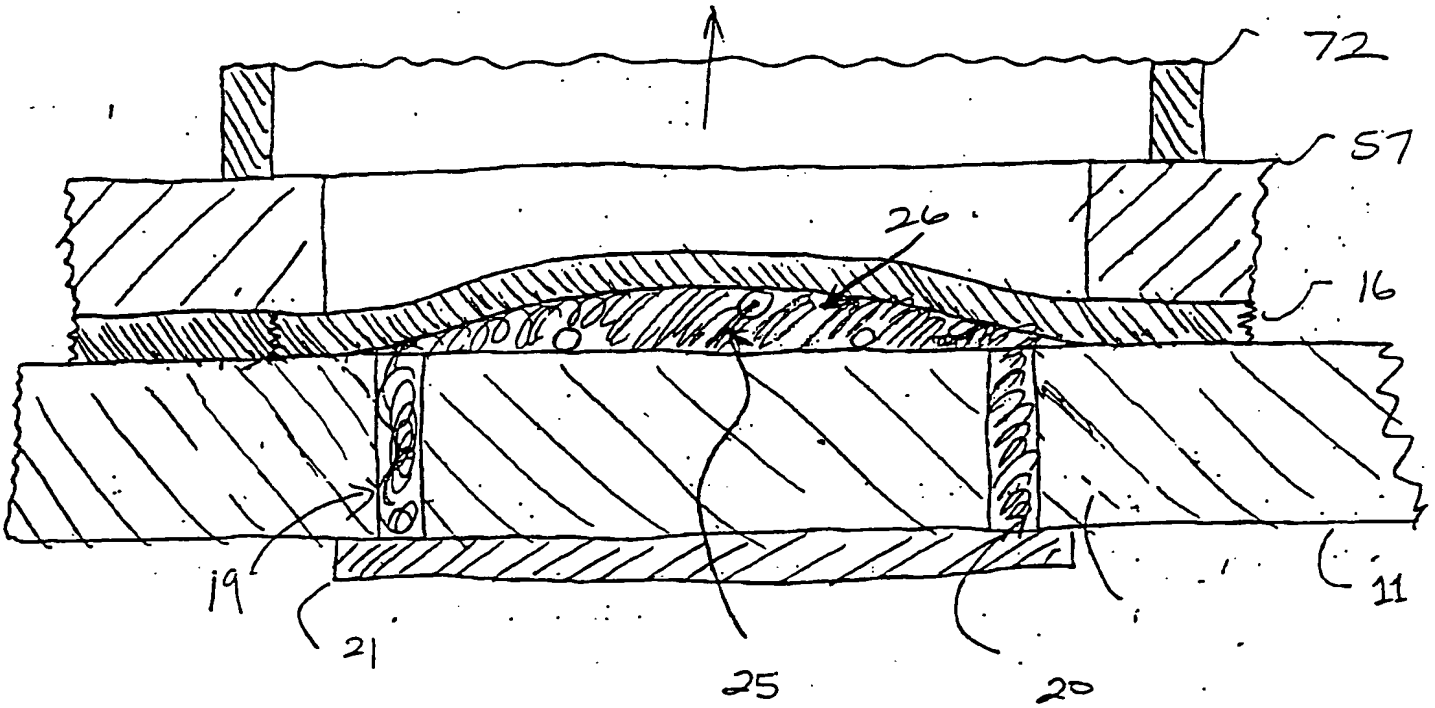


FIG. 14

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International Bureau

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09/492,013 26 January 2000 (26.01.2000) US

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Filed on 26 January 2000 (26.01.2000)

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(72) Inventors; and

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(74) Agents: **SILVA, Robin, M.** et al.; Flehr Hohbach Test Albritton & Herbert LLP, 4 Embarcadero Center, Suite 3400, San Francisco, CA 94111-4187 (US).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*

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14 February 2002

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/54814 A3

(54) Title: COMPOSITIONS AND METHODS FOR PERFORMING BIOLOGICAL REACTIONS

(57) Abstract: The present invention relates to an apparatus for performing biological reactions. Specifically, the invention relates to an apparatus for performing nucleic acid hybridization reactions on a substrate layer having a multiplicity of oligonucleotide binding sites disposed thereon, using substrates with flexible covers.

INTERNATIONAL SEARCH REPORT

Inter onal Application No
PCT/US 01/02664

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 B01L3/00 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 B01L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 922 591 A (FODOR STEPHEN P A ET AL) 13 July 1999 (1999-07-13) cited in the application column 2, line 29 - line 34 column 6, line 17 - line 24	1-5,8-11
X	column 8, line 51 - line 54	5
Y	column 29, line 47 -column 30, line 12; figure 12A column 32, line 30 - line 57; figure 7A	7
Y	column 35, line 25 - line 43	6
Y	column 37, line 4 - line 32 ---	6
Y	US 3 429 796 A (LAUER JAY M) 25 February 1969 (1969-02-25) column 3, line 15 - line 20 ---	7
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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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Date of the actual completion of the international search	Date of mailing of the international search report
29 August 2001	05/09/2001

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European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Hocquet, A

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INTERNATIONAL SEARCH REPORT

Inter ional Application No
PCT/US 01/02664

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 97 16561 A (SARNOFF DAVID RES CENTER) 9 May 1997 (1997-05-09) cited in the application page 11, line 22 - line 26; figure 4A	8,9
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3

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter onal Application No
PCT/US 01/02664

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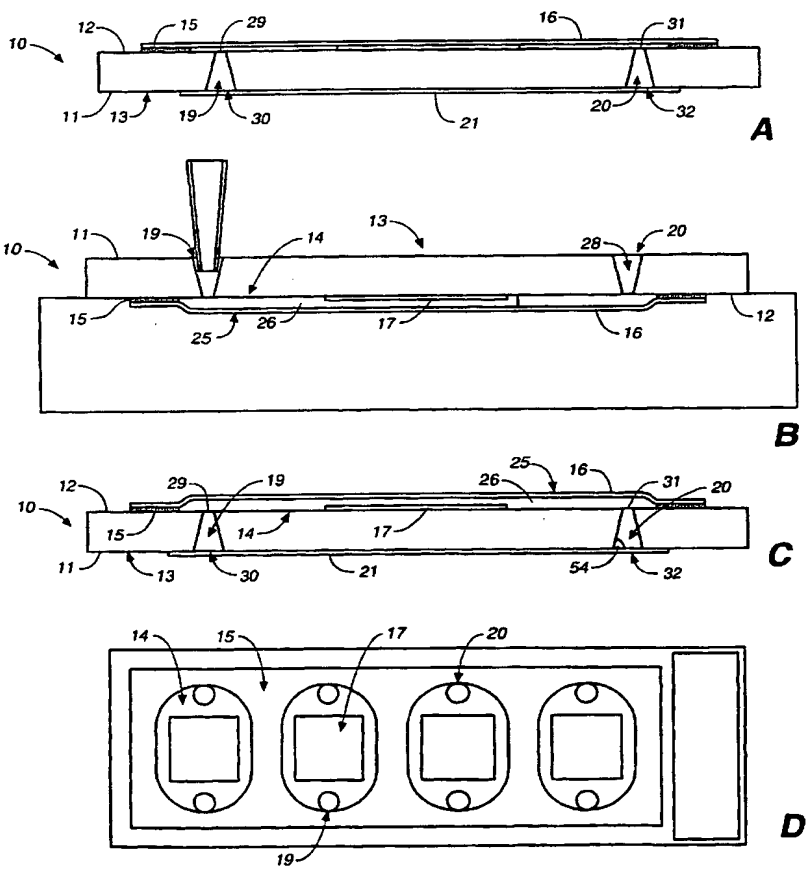
(54) Title: COMPOSITIONS AND METHODS FOR PERFORMING BIOLOGICAL REACTIONS



(57) Abstract: The present invention relates to an apparatus for performing biological reactions. Specifically, the invention relates to an apparatus for performing nucleic acid hybridization reactions on a substrate layer having a multiplicity of oligonucleotide binding sites disposed thereon, using substrates with flexible covers.

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COMPOSITIONS AND METHODS FOR PERFORMING BIOLOGICAL REACTIONS

This application is a continuing application of U.S.S.N. 09/492,013, filed January 26, 2000.

FIELD OF THE INVENTION

The present invention relates to an apparatus for performing biological reactions. In particular, the invention relates to an apparatus for performing nucleic acid hybridization reactions on a substrate layer having a multiplicity of oligonucleotide binding sites disposed thereon, using substrates with flexible covers, and a method for removing gas bubbles from the apparatus. Specifically, the invention relates to an apparatus having a flexible, gas permeable layer affixed to a substrate layer with an adhesive, wherein the flexible, gas permeable layer, the adhesive and the substrate layer enclose a reaction chamber, and a means for facilitating diffusion across the flexible, gas permeable layer. The diffusion-facilitating means creates a pressure gradient or concentration gradient across the flexible, gas permeable layer, thereby increasing the rate of diffusion of gas molecules from the reaction chamber across the flexible, gas permeable layer.

BACKGROUND OF THE INVENTION

Recent advances in molecular biology have provided the opportunity to identify pathogens, diagnose disease states, and perform forensic determinations using gene sequences specific for the desired purpose. This explosion of genetic information has created a need for high-capacity assays and equipment for performing molecular biological assays, particularly nucleic acid hybridization assays. Most urgently, there is a need to miniaturize, automate, standardize and simplify such assays. This need stems from the fact that while these hybridization assays were originally developed in research laboratories working with purified products and performed by highly skilled individuals, adapting these procedures to clinical uses, such as diagnostics, forensics and other applications, has produced the need for equipment and methods that allow less-skilled operators to effectively perform the assays under higher capacity, less stringent assay conditions.

Existing technology utilizes the binding of molecules contained within a biologically reactive sample

fluid, hereinafter referred to as target molecules, onto molecules contained within biologically reactive sites, hereinafter referred to as probe molecules. The primary enabler of this technology is an apparatus commonly referred to as a biochip, which comprises one or more ordered microscopic arrays ("microarrays") of biologically reactive sites immobilized on the surface of a substrate. A biologically reactive site can be created by dispensing a small volume of a fluid containing a biological reagent onto a discrete location on the surface of a substrate, also commonly referred to as spotting. To enhance immobilization of probe molecules, biochips can include a 2-dimensional array of 3-dimensional polymeric anchoring structures (for example, polyacrylamide gel pads) attached to the surface of the substrate. Probe molecules such as oligonucleotides are covalently attached to polyacrylamide-anchoring structures by forming amide, ester or disulfide bonds between the biomolecule and a derivatized polymer comprising the cognate chemical group. Covalent attachment of probe molecules to such polymeric anchoring structures is usually performed after polymerization and chemical cross-linking of the polymer to the substrate is completed.

Biochips are advantageously used to perform biological reactions on the surface thereof. Existing apparatus for performing biological reactions on a substrate surface, however, are deficient in that they either require unacceptably large volumes of sample fluid to operate properly, cannot accommodate substrates as large as or larger than a conventional microscope slide, cannot independently accommodate a plurality of independent reactions, or cannot accommodate a substrate containing hydrogel-based microarrays. Most existing apparatus also do not allow introduction of fluids in addition to the sample fluid (such as wash buffers, fluorescent dyes, etc.) into the reaction chamber. Disposable apparatus must be disassembled and reassembled around the biochip every time a new fluid must be introduced. Other existing apparatus are difficult to use in a laboratory environment because they cannot be loaded with standard pipet tips and associated pipettor apparatus.

Many existing apparatus also exhibit unacceptable reaction reproducibility, efficiency, and duration. Reaction reproducibility may be adversely affected by bubble formation in the reaction chamber or by the use of biologically incompatible materials for the reaction chamber. Reaction duration and efficiency may be adversely affected by the presence of concentration gradients in the reaction chamber.

Bubbles can form upon introduction of sample fluid to the reaction chamber or by outgassing of the reaction chamber materials. When gas bubbles extend over the substrate surface in an area containing biologically reactive sites, the intended reaction may intermittently fail or yield erroneous results because the intended concentration of the sample fluid mixture has been compromised by the presence of gas bubbles.

Biologically incompatible reaction chamber materials may cause unacceptable reaction reproducibility,

by interacting with the sample fluid, thus causing the intended reaction to intermittently fail or yield erroneous results.

Incomplete mixing of the sample fluid can introduce concentration gradients within the sample fluid that adversely impact reaction efficiency and duration. This effect is most pronounced when there is a depletion of target molecules in the local volume surrounding a biologically reactive site. During a biological reaction, the probability that a particular target molecule will bind to a complementary (immobilized) probe molecule is determined by the given concentration of target molecules present within the sample fluid volume, the diffusion rate of the target molecule through the reaction chamber, and the statistics of interaction between the target molecule and the complementary probe molecule. For diagnostic assays, target DNA molecules are often obtained in minute (< picomol) quantities. In practice, it can take tens of hours for a hybridization reaction to be substantially complete at the low target nucleic acid molecule levels available for biological samples. Concentration gradients in the hybridization chamber can further exacerbate this problem.

U.S. Patent 5,948,673 to Cottingham discloses a self-contained multi-chamber reactor for performing both DNA amplification and DNA probe assays in a sealed unit wherein some reactants are provided by coating the walls of the chambers and other reactants are introduced into the chambers prior to starting the reaction in order to eliminate flow into and out of the chamber. No provisions are made for eliminating gas bubbles from the chambers.

There remains a need in the art for methods and apparatus for performing biological reactions on a substrate surface that use a low volume of sample fluid, that accommodate substrates as large as or larger than a conventional microscope slide, that accommodate a plurality of independent reactions, and that accommodate a substrate surface having one or more hydrogel-based microarrays attached thereto. There also remains a need in the art for an apparatus that allows introduction of fluids in addition to sample fluid into each reaction chamber via standard pipet tips and associated pipettor apparatus. There also remains a need in the art for such an apparatus that increases reaction reproducibility, increases reaction efficiency, and reduces reaction duration. There also remains a need in this art for a simple method for removing gas bubbles from such an apparatus. These needs are particularly striking in view of the tremendous interest in biochip technology, the investment and substantial financial rewards generated by research into biochip technology, and the variety of products generated by such research.

Nucleic acid hybridization assays are advantageously performed using probe array technology, which utilizes binding of target single-stranded DNA onto immobilized DNA (usually, oligonucleotide) probes. The detection limit of a nucleic acid hybridization assay is determined by the sensitivity of the detection device, and also by the amount of target nucleic acid available to be bound to probes, typically oligonucleotide probes, during hybridization.

Nucleic acid hybridization chambers are known in the prior art. U.S. Patent No. 5,100,755 to Smyczek et al. discloses a hybridization chamber. U.S. Patent No. 5,545,531 to Rava et al. discloses a hybridization plate comprising a multiplicity of oligonucleotide arrays. U.S. Patent No. 5,360,741 to Hunnell discloses a gas heated hybridization chamber. U.S. Patent No. 5,922,591 to Anderson et al. discloses a miniaturized hybridization chamber for use with oligonucleotide arrays. U.S. Patent No. 5,945,334 to Besemer discloses oligonucleotide array packaging.

As currently employed, oligonucleotide array technology does not provide maximum hybridization efficiency. Existing nucleic acid hybridization assay equipment includes numerous components, each of which is a source of inefficiency and inaccuracy.

Hybridization using oligonucleotide arrays must be performed in a volume in which a small amount of target DNA or other nucleic acid can be efficiently annealed to the immobilized probes. For diagnostic assays, target DNA molecules are often obtained in minute (< picomol) quantities. In practice, it can take several (tens of) hours for hybridization to be substantially complete at the low target nucleic acid levels available for biological samples.

In addition, array hybridization is conventionally performed in a stationary hybridization chamber where active mixing is absent. Under these conditions, the probability that a particular target molecule will hybridize to a complementary oligonucleotide probe immobilized on a surface is determined by the concentration of the target, the diffusion rate of the target molecule and the statistics of interaction between the target and the complementary oligonucleotide. Consequently, a larger number of samples must be tested to obtain useful information, and this in turn leads to increased hybridization times and inefficiencies.

In addition, efficiency is increased when the amount of user manipulation is kept to a minimum. As currently performed, oligonucleotide array hybridization requires a great deal of operator attentiveness and manipulation, and the degree of skill required to perform the analysis is high. For example, hybridization is typically performed in an assay chamber, and then data collection and analysis are performed in a separate apparatus (such as a laser scanner or fluorescence microscope). This arrangement requires a substantial amount of handling by the user, and makes the assays both time-consuming and subject to user error.

It is also a limitation of current practice that array hybridizations are performed one array at a time, thereby forgoing the economies of parallel processing and data analysis.

Additional limitations, inefficiencies, and expenses arise from the structural characteristics of existing apparatus. Many existing apparatus are limited in the size of the substrate they can accommodate. Other apparatus are not disposable and therefore require extensive cleaning between runs in order to

prevent sample contamination. Yet other apparatus are high mass and therefore not susceptible of the rapid heating and cooling necessary for efficient hybridization. Other apparatus require the use of expensive optics for analysis of the reaction products.

There remains a need in this art for an easy-to-use apparatus for performing biological reactions, particularly nucleic acid hybridization, that comprises a small reaction volume, where the fluid components can be actively mixed, that can be performed in parallel and that minimizes user intervention. There also remains a need for such an apparatus that is easy to manufacture in various sizes, that is disposable to minimize sample contamination, that allows for the use of low cost optical analytical equipment, and that is low mass to allow for rapid heating and cooling of the sample fluid. There also remains a need for methods for using such apparatus to increase hybridization efficiency, particularly relating to biochip arrays as understood in the art. This need is particularly striking, in view of the tremendous interest in biochip technology, the investment and substantial financial rewards generated by research into biochip technology, and the variety of products generated by such research.

SUMMARY OF THE INVENTION

In accordance with the objects outlined above, the present invention provides apparatus for performing biological reactions, comprising a substrate that has a first and a second surface (usually opposite to each other, in the case of planar substrates). An array of biomolecules are positioned on the first surface, and a flexible layer affixed to the first surface of the substrate by an adhesive layer, wherein the adhesive layer is deposited on the first surface and forms a reaction volume. The substrate comprises at least a first port extending through the substrate from the first to the second surface, and the port has a first opening and a second opening. The first opening of the port is provided within the area bounded by the adhesive and covered by the flexible layer (e.g. the reaction volume) and the second opening of the port is provided on the second surface of the substrate. Alternatively, the port(s) may be in the flexible layer into the reaction volume.

Optionally, there may be a removable cover positioned over the second opening of the port such as a foil tape, as well as a layer of a water-soluble compound that is a solid at a first temperature and a liquid at a second, higher temperature, the layer being positioned between the first surface of the substrate and the flexible layer. Optionally, the flexible layer is a translucent plastic or gas permeable membrane.

In a further aspect, the invention provides an apparatus for performing biological reactions on a substrate surface and a method for removing gas bubbles from the apparatus to prevent interference with biological reactions such as hybridization at reaction sites on the substrate surface. Specifically, the method of the invention is directed to an apparatus comprising a flexible, gas permeable layer affixed to a biochip with an adhesive, wherein the flexible, gas permeable layer, the adhesive, and the

biochip enclose a reaction chamber, and a means for facilitating diffusion of gas molecules out of the reaction chamber across the flexible, gas permeable layer. The diffusion-facilitating means, referred to herein as a gas diffusion accelerator, creates a pressure gradient or concentration gradient across the flexible, gas permeable layer, thereby increasing the rate of diffusion of gas molecules from the reaction chamber through the flexible, gas permeable layer.

The port can be in the shape of a truncated cone having a small-diameter end and a large diameter end, and wherein the small diameter end is provided in the area on the first substrate surface bounded by the adhesive layer (the reaction volume) and the large diameter end is provided on the second substrate surface. The walls of a port in some embodiments form an angle of less than 90° with the second substrate surface. Optionally, the port contains a sample fluid having a contact angle, and the angle formed between the second substrate surface and the port walls is less than or equal to the contact angle of the sample fluid.

A second port can be included; in this embodiment, the second port extends through the substrate from the first surface to the second surface thereof and having a first opening and a second opening, wherein the first opening of the outlet port is provided on the first substrate surface within the area bounded by the adhesive and covered by the flexible layer, and the second opening of the outlet port is provided on the second substrate surface and is covered by a removable cover. Alternatively, the second port is in the flexible layer as above.

Optionally, the apparatus of the invention can comprise a reflective layer positioned between the array and the first substrate surface, and/or a resistive heater; the latter can be a reflective layer positioned between the array and the first substrate surface.

Additionally, the apparatus can comprise a passivation layer such as parylene, or a scanner, wherein the scanner is in contact with the flexible layer at a position over the array. The scanner can be a light pipe and cover the entirety of the reaction volume.

In a further aspect, the apparatus further comprises a sample preparation chip in contact with the second substrate surface wherein the sample preparation chip has a port that is aligned with the first port of the apparatus.

In an additional aspect, the apparatus further comprises a roller, wherein the roller is in contact with the flexible layer at a position over the array; for example, it can move longitudinally across the reaction volume.

In a further aspect, the apparatus can include a case having a lid and a base with a cavity, and a carriage comprising a scanner and a roller, wherein the carriage is provided in the cavity, wherein the

substrate is removably positioned above the carriage such that the first substrate surface is in operative contact with the carriage.

In an additional aspect, the invention provides apparatus for performing biological reactions, comprising a glass microscope slide having a first surface and a second surface opposite thereto; an array of biomolecules positioned on the first surface of the slide, wherein each biomolecule within the array is anchored to the first surface by a polyacrylamide gel pad; a layer of polyvinylidene chloride affixed to the first surface of the slide by a layer of double-sided adhesive, wherein the adhesive layer is deposited on the first surface of the slide and encloses an area thereupon; a first and second port extending through the slide from the first surface to the second surface thereof each having a first opening and a second opening, wherein the first opening of each port is provided within the area on the first surface of the slide bounded by the adhesive and covered by the flexible layer and the second opening of each port is provided on the second surface of the slide, and wherein each port is in the shape of a truncated cone having a small-diameter end and a large diameter end, and wherein the small diameter end is the first opening and the large diameter end is the second opening; a layer of foil tape positioned over the second opening of each port; a layer of a polyethylene glycol positioned between the first surface of the slide and the layer of polyvinylidene chloride; a reflective layer positioned between the array and the first substrate surface; a layer of parylene positioned between the reflective layer and the and the layer of polyvinylidene chloride; and a resistive heater.

In a further aspect, the invention provides an apparatus for performing biological reactions, comprising a substrate having a first surface and a second surface, a multiplicity of biomolecules positioned on the first surface of the substrate, a flexible layer affixed to the first surface of the substrate by an adhesive layer, wherein the adhesive layer is deposited on the first surface of the substrate and encloses an area thereupon, and wherein a reaction volume is enclosed between the flexible layer and the first substrate surface in the area defined by the adhesive layer. There are first and second ports extending through the flexible layer and the adhesive layer into the volume enclosed between the flexible layer and the first substrate surface in the area defined by the adhesive layer.

In an additional aspect, each of the multiplicity of biomolecules is attached to an anchoring structure such as a gel pad of a polymeric material such as polyacrylamide, either as discrete sites or as a continuous layer.

In a further aspect, the apparatus comprises a label layer affixed to the flexible layer, wherein the first and second ports extend through the label layer and the flexible layer. The label layer can comprise an adhesive surface and a non-adhesive surface, and wherein the label layer is affixed to the flexible layer using the adhesive surface. The label layer can comprise a window corresponding in size and position to the area bounded by the adhesive layer, and wherein the window allows visual inspection of the flexible layer and the volume enclosed between the flexible layer and the first substrate surface in

the area defined by the adhesive layer. Optionally, a reflective layer positioned between the array and the first substrate surface is provided, and/or a resistive heater.

In an additional aspect, the invention provides methods of using the apparatus to detect target analytes.

BRIEF DESCRIPTION OF THE DRAWINGS

Presently preferred embodiments of the invention are described with reference to the following drawings.

FIGS. **1A-1D** are views of a preferred embodiment of the present invention illustrating the preparation of a chamber for reaction. FIG. **1A** is a cross-sectional view of the apparatus illustrating a reaction chamber prefilled with a water-soluble compound in thermal contact with a heating element. FIG. **1B** is a cross sectional view of the apparatus illustrating the mixing of the water-soluble compound and the biological sample fluid. FIG. **1C** is a cross sectional view of the apparatus illustrating a chamber filled with the sample fluid/water-soluble compound mixture, wherein the first and second ports are covered with a seal. FIG. **1D** is a top plan view of the apparatus illustrating the pattern of adhesive defining the individual areas containing the arrays of oligonucleotide probes.

FIG. **2** is an exploded cross-sectional view of a chamber showing the array of gel pads of a preferred embodiment of the invention.

FIG. **3** is an exploded perspective view of the array of biomolecular probes showing the positioning of the gel pads on the substrate of a preferred embodiment of the invention.

FIG. **4** is an exploded cross-sectional view of a port illustrating the conical shape of the port of a preferred embodiment of the invention.

FIG. **5** is a perspective view of the label layer, the flexible layer and the adhesive layer of a preferred embodiment of the invention.

FIG. **6** is a cross-sectional view of a stack of chambers according to a preferred embodiment.

FIGS. **7A-7E** are top views of the layers of an alternate preferred embodiment of the invention having inlet and outlet ports extending through the flexible layer. FIG. **7A** is a view of the first adhesive layer, FIG. **7B** is a view of the flexible layer, FIG. **7C** is a view of the second adhesive layer, FIG. **7D** is a view of the label layer, and FIG. **7E** is a view of the layers of **7A** to **7D** as assembled.

FIGS. **8A-8B** are detail views of the notches cut into the first adhesive layer and the label layer of a preferred embodiment of the invention having inlet and outlet ports extending through the flexible layer.

FIGS. **9A-9C** are cross-sectional views of a preferred embodiment of the present invention illustrating the process of analyzing the array after completion of the reaction. FIG. **9A** shows the apparatus upon completion of the reaction. FIG. **9B** illustrates removal of the sample fluid from the chamber such that the flexible layer contacts the array. FIG. **9C** illustrates use of a laser scanner to analyze the array.

FIGS. **10A-10C** illustrate a handheld embodiment of the present invention. FIG. **10A** is a side view of the hand held scanning system. FIG. **10B** is a perspective view of a preferred embodiment comprising a hand-held scanning device illustrating the contact of the flexible layer with the carriage. FIG. **10C** is a view of the handheld system illustrating the lid containing the display unit.

FIG. **11A-11E** are cross-sectional views of the direct contact fiber optic scanner as shown in FIG. **10**.

FIG **12A-12C** are alternate embodiments illustrating the apparatus coupled to a sample preparation chip. FIG. **12A** illustrates an embodiment wherein the sample preparation chip is removably positioned against the second surface of the substrate. FIG. **12B** illustrates an embodiment wherein the sample preparation chip is affixed to the second surface of the substrate. FIG. **12C** illustrates an embodiment wherein the sample preparation chip is incorporated into the substrate.

FIG. **13** illustrates the assembly and use of a preferred embodiment of the present invention.

Figure 14 depicts a cross sectional view of a preferred embodiment of the present invention illustrating the application of vacuum to a reaction chamber or volume.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to methods and compositions for performing high capacity biological reactions on a biochip comprising a substrate having an array of biological binding sites. The invention provides a reaction chamber, such as a reaction chamber in the case where the biochip comprises nucleic acids. The reaction chamber is formed with a substrate, a layer of adhesive and a flexible cover. The system utilizes ports, either in the substrate or in the flexible cover, to allow sample and/or reagent loading. In addition, the invention provides methods for removing gas bubbles from the apparatus using a gas diffusion accelerator, that will facilitate and accelerate the rate of diffusion through the gas permeable, flexible membrane.

Accordingly, the present invention provides devices of the invention are used to detect target analytes

in samples. By "target analyte" or "analyte" or grammatical equivalents herein is meant any molecule, compound or particle to be detected. As outlined below, target analytes preferably bind to binding ligands, as is more fully described above. As will be appreciated by those in the art, a large number of analytes may be detected using the present methods; basically, any target analyte for which a binding ligand, described herein, may be made may be detected using the methods of the invention.

Suitable analytes include organic and inorganic molecules, including biomolecules. In a preferred embodiment, the analyte may be an environmental pollutant (including pesticides, insecticides, toxins, etc.); a chemical (including solvents, polymers, organic materials, etc.); therapeutic molecules (including therapeutic and abused drugs, antibiotics, etc.); biomolecules (including hormones, cytokines, proteins, lipids, carbohydrates, cellular membrane antigens and receptors (neural, hormonal, nutrient, and cell surface receptors) or their ligands, etc); whole cells (including procaryotic (such as pathogenic bacteria) and eukaryotic cells, including mammalian tumor cells); viruses (including retroviruses, herpesviruses, adenoviruses, lentiviruses, etc.); and spores; etc. Particularly preferred analytes are environmental pollutants; nucleic acids; proteins (including enzymes, antibodies, antigens, growth factors, cytokines, etc); therapeutic and abused drugs; cells; and viruses.

In a preferred embodiment, the target analyte is a nucleic acid. By "nucleic acid" or "oligonucleotide" or grammatical equivalents herein means at least two nucleotides covalently linked together. A nucleic acid of the present invention will generally contain phosphodiester bonds, although in some cases, as outlined below, nucleic acid analogs are included that may have alternate backbones, comprising, for example, phosphoramide (Beaucage et al., Tetrahedron 49(10):1925 (1993) and references therein; Letsinger, J. Org. Chem. 35:3800 (1970); Sprinzl et al., Eur. J. Biochem. 81:579 (1977); Letsinger et al., Nucl. Acids Res. 14:3487 (1986); Sawai et al, Chem. Lett. 805 (1984), Letsinger et al., J. Am. Chem. Soc. 110:4470 (1988); and Pauwels et al., Chemica Scripta 26:141 91986)), phosphorothioate (Mag et al., Nucleic Acids Res. 19:1437 (1991); and U.S. Patent No. 5,644,048), phosphorodithioate (Briu et al., J. Am. Chem. Soc. 111:2321 (1989), O-methylphophoroamidite linkages (see Eckstein, Oligonucleotides and Analogues: A Practical Approach, Oxford University Press), and peptide nucleic acid backbones and linkages (see Egholm, J. Am. Chem. Soc. 114:1895 (1992); Meier et al., Chem. Int. Ed. Engl. 31:1008 (1992); Nielsen, Nature, 365:566 (1993); Carlsson et al., Nature 380:207 (1996), all of which are incorporated by reference). Other analog nucleic acids include those with positive backbones (Denpcy et al., Proc. Natl. Acad. Sci. USA 92:6097 (1995); non-ionic backbones (U.S. Patent Nos. 5,386,023, 5,637,684, 5,602,240, 5,216,141 and 4,469,863; Kiedrowshi et al., Angew. Chem. Intl. Ed. English 30:423 (1991); Letsinger et al., J. Am. Chem. Soc. 110:4470 (1988); Letsinger et al., Nucleoside & Nucleotide 13:1597 (1994); Chapters 2 and 3, ASC Symposium Series 580, "Carbohydrate Modifications in Antisense Research", Ed. Y.S. Sanghui and P. Dan Cook; Mesmaeker et al., Bioorganic & Medicinal Chem. Lett. 4:395 (1994); Jeffs et al., J. Biomolecular NMR 34:17 (1994); Tetrahedron Lett. 37:743 (1996)) and non-ribose backbones, including those described in U.S. Patent Nos. 5,235,033 and 5,034,506, and

Chapters 6 and 7, ASC Symposium Series 580, "Carbohydrate Modifications in Antisense Research", Ed. Y.S. Sanghui and P. Dan Cook. Nucleic acids containing one or more carbocyclic sugars are also included within the definition of nucleic acids (see Jenkins et al., Chem. Soc. Rev. (1995) pp169-176). Several nucleic acid analogs are described in Rawls, C & E News June 2, 1997 page 35. Nucleic acid analogs also include "locked nucleic acids". All of these references are hereby expressly incorporated by reference. These modifications of the ribose-phosphate backbone may be done to facilitate the addition of electron transfer moieties, or to increase the stability and half-life of such molecules in physiological environments.

As will be appreciated by those in the art, all of these nucleic acid analogs may find use in the present invention. In addition, mixtures of naturally occurring nucleic acids and analogs can be made; for example, at the site of conductive oligomer or electron transfer moiety attachment, an analog structure may be used. Alternatively, mixtures of different nucleic acid analogs, and mixtures of naturally occurring nucleic acids and analogs may be made.

As outlined herein, the nucleic acids may be single stranded or double stranded, as specified, or contain portions of both double stranded or single stranded sequence. The nucleic acid may be DNA, both genomic and cDNA, RNA or a hybrid, where the nucleic acid contains any combination of deoxyribo- and ribo-nucleotides, and any combination of bases, including uracil, adenine, thymine, cytosine, guanine, inosine, xathanine hypoxathanine, isocytosine, isoguanine, etc. As used herein, the term "nucleoside" includes nucleotides and nucleoside and nucleotide analogs, and modified nucleosides such as amino modified nucleosides. In addition, "nucleoside" includes non-naturally occuring analog structures. Thus for example the individual units of a peptide nucleic acid, each containing a base, are referred to herein as nucleosides.

In a preferred embodiment, the present invention provides methods of detecting target nucleic acids. By "target nucleic acid" or "target sequence" or grammatical equivalents herein means a nucleic acid sequence on a single strand of nucleic acid. The target sequence may be a portion of a gene, a regulatory sequence, genomic DNA, cDNA, RNA including mRNA and rRNA, or others. It may be any length, with the understanding that longer sequences are more specific. In some embodiments, it may be desirable to fragment or cleave the sample nucleic acid into fragments of 100 to 10,000 basepairs, with fragments of roughly 500 basepairs being preferred in some embodiments. As will be appreciated by those in the art, the complementary target sequence may take many forms. For example, it may be contained within a larger nucleic acid sequence, i.e. all or part of a gene or mRNA, a restriction fragment of a plasmid or genomic DNA, among others.

As is outlined more fully below, probes (including primers) are made to hybridize to target sequences to determine the presence or absence of the target sequence in a sample. Generally speaking, this term will be understood by those skilled in the art.

The target sequence may also be comprised of different target domains, which may be adjacent (i.e. contiguous) or separated. For example, when ligation chain reaction (LCR) techniques are used, a first primer may hybridize to a first target domain and a second primer may hybridize to a second target domain; either the domains are adjacent, or they may be separated by one or more nucleotides, coupled with the use of a polymerase and dNTPs, as is more fully outlined below. The terms "first" and "second" are not meant to confer an orientation of the sequences with respect to the 5'-3' orientation of the target sequence. For example, assuming a 5'-3' orientation of the complementary target sequence, the first target domain may be located either 5' to the second domain, or 3' to the second domain.

In a preferred embodiment, the target analyte is a protein. As will be appreciated by those in the art, there are a large number of possible proteinaceous target analytes that may be detected using the present invention. By "proteins" or grammatical equivalents herein is meant proteins, oligopeptides and peptides, derivatives and analogs, including proteins containing non-naturally occurring amino acids and amino acid analogs, and peptidomimetic structures. The side chains may be in either the (R) or the (S) configuration. In a preferred embodiment, the amino acids are in the (S) or L-configuration. As discussed below, when the protein is used as a binding ligand, it may be desirable to utilize protein analogs to retard degradation by sample contaminants.

Suitable protein target analytes include, but are not limited to, (1) immunoglobulins, particularly IgEs, IgGs and IgMs, and particularly therapeutically or diagnostically relevant antibodies, including but not limited to, for example, antibodies to human albumin, apolipoproteins (including apolipoprotein E), human chorionic gonadotropin, cortisol, α-fetoprotein, thyroxin, thyroid stimulating hormone (TSH), antithrombin, antibodies to pharmaceuticals (including antieptileptic drugs (phenytoin, primidone, carbariezepin, ethosuximide, valproic acid, and phenobarbitol), cardioactive drugs (digoxin, lidocaine, procainamide, and disopyramide), bronchodilators (theophylline), antibiotics (chloramphenicol, sulfonamides), antidepressants, immunosuppresants, abused drugs (amphetamine, methamphetamine, cannabinoids, cocaine and opiates) and antibodies to any number of viruses (including orthomyxoviruses, (e.g. influenza virus), paramyxoviruses (e.g respiratory syncytial virus, mumps virus, measles virus), adenoviruses, rhinoviruses, coronaviruses, reoviruses, togaviruses (e.g. rubella virus), parvoviruses, poxviruses (e.g. variola virus, vaccinia virus), enteroviruses (e.g. poliovirus, coxsackievirus), hepatitis viruses (including A, B and C), herpesviruses (e.g. Herpes simplex virus, varicella-zoster virus, cytomegalovirus, Epstein-Barr virus), rotaviruses, Norwalk viruses, hantavirus, arenavirus, rhabdovirus (e.g. rabies virus), retroviruses (including HIV, HTLV-I and -II), papovaviruses (e.g. papillomavirus), polyomaviruses, and picornaviruses, and the like), and bacteria (including a wide variety of pathogenic and non-pathogenic prokaryotes of interest including Bacillus; Vibrio, e.g. *V. cholerae;* Escherichia, e.g. Enterotoxigenic *E. coli*, Shigella, e.g. *S. dysenteriae;* Salmonella, e.g. *S. typhi;* Mycobacterium e.g. *M. tuberculosis, M. leprae*; Clostridium, e.g. *C. botulinum, C. tetani, C. difficile, C.perfringens*; Cornyebacterium, e.g. *C. diphtheriae*;

Streptococcus, *S. pyogenes, S. pneumoniae;* Staphylococcus, *e.g. S. aureus*; Haemophilus, e.g. *H. influenzae*; Neisseria, e.g. *N. meningitidis, N. gonorrhoeae*; Yersinia, e.g. *G. lamblia Y. pestis*, Pseudomonas, e.g. *P. aeruginosa, P. putida*; Chlamydia, e.g. *C. trachomatis*; Bordetella, e.g. *B. pertussis;* Treponema, e.g. *T. palladium;* and the like); (2) enzymes (and other proteins), including but not limited to, enzymes used as indicators of or treatment for heart disease, including creatine kinase, lactate dehydrogenase, aspartate amino transferase, troponin T, myoglobin, fibrinogen, cholesterol, triglycerides, thrombin, tissue plasminogen activator (tPA); pancreatic disease indicators including amylase, lipase, chymotrypsin and trypsin; liver function enzymes and proteins including cholinesterase, bilirubin, and alkaline phosphotase; aldolase, prostatic acid phosphatase, terminal deoxynucleotidyl transferase, and bacterial and viral enzymes such as HIV protease; (3) hormones and cytokines (many of which serve as ligands for cellular receptors) such as erythropoietin (EPO), thrombopoietin (TPO), the interleukins (including IL-1 through IL-17), insulin, insulin-like growth factors (including IGF-1 and -2), epidermal growth factor (EGF), transforming growth factors (including TGF-α and TGF-β), human growth hormone, transferrin, epidermal growth factor (EGF), low density lipoprotein, high density lipoprotein, leptin, VEGF, PDGF, ciliary neurotrophic factor, prolactin, adrenocorticotropic hormone (ACTH), calcitonin, human chorionic gonadotropin, cotrisol, estradiol, follicle stimulating hormone (FSH), thyroid-stimulating hormone (TSH), leutinzing hormone (LH), progeterone and testosterone; and (4) other proteins (including α-fetoprotein, carcinoembryonic antigen CEA, cancer markers, etc.).

In addition, any of the biomolecules for which antibodies may be detected may be detected directly as well; that is, detection of virus or bacterial cells, therapeutic and abused drugs, etc., may be done directly.

Suitable target analytes include carbohydrates, including but not limited to, markers for breast cancer (CA15-3, CA 549, CA 27.29), mucin-like carcinoma associated antigen (MCA), ovarian cancer (CA125), pancreatic cancer (DE-PAN-2), prostate cancer (PSA), CEA, and colorectal and pancreatic cancer (CA 19, CA 50, CA242).

Suitable target analytes include metal ions, particularly heavy and/or toxic metals, including but not limited to, aluminum, arsenic, cadmium, selenium, cobalt, copper, chromium, lead, silver and nickel.

These target analytes may be present in any number of different sample types, including, but not limited to, bodily fluids including blood, lymph, saliva, vaginal and anal secretions, urine, feces, perspiration and tears, and solid tissues, including liver, spleen, bone marrow, lung, muscle, brain, etc.

Accordingly, the present invention provides devices for the detection of target analytes comprising a solid substrate. The solid substrate can be made of a wide variety of materials and can be configured

in a large number of ways, as is discussed herein and will be apparent to one of skill in the art. In addition, a single device may be comprises of more than one substrate; for example, there may be a "sample treatment" cassette that interfaces with a separate "detection" cassette; a raw sample is added to the sample treatment cassette and is manipulated to prepare the sample for detection, which is removed from the sample treatment cassette and added to the detection cassette. There may be an additional functional cassette into which the device fits; for example, a heating element which is placed in contact with the sample cassette to effect reactions such as PCR. In some cases, a portion of the substrate may be removable; for example, the sample cassette may have a detachable detection cassette, such that the entire sample cassette is not contacted with the detection apparatus. See for example U.S. Patent No. 5,603,351, PCT US96/17116, and "MULTILAYERED MICROFLUIDIC DEVICES FOR ANALYTE REACTIONS" filed in the PCT December 11, 2000, Serial No. PCT/US00/33499, hereby incorporated by reference.

The composition of the solid substrate will depend on a variety of factors, including the techniques used to create the device, the use of the device, the composition of the sample, the analyte to be detected, the size of the wells and microchannels, the presence or absence of electronic components, etc. Generally, the devices of the invention should be easily sterilizable as well.

In a preferred embodiment, the solid substrate can be made from a wide variety of materials including, but not limited to, silicon such as silicon wafers, silcon dioxide, silicon nitride, glass and fused silica, gallium arsenide, indium phosphide, aluminum, ceramics, polyimide, quartz, plastics, resins and polymers including polymethylmethacrylate, acrylics, polyethylene, polyethylene terepthalate, polycarbonate, polystyrene and other styrene copolymers, polypropylene, polytetrafluoroethylene, superalloys, zircaloy, steel, gold, silver, copper, tungsten, molybdeumn, tantalum, KOVAR, KEVLAR, KAPTON, MYLAR, brass, sapphire, etc. High quality glasses such as high melting borosilicate or fused silicas may be preferred for their UV transmission properties when any of the sample manipulation steps require light based technologies. In addition, as outlined herein, portions of the internal surfaces of the device may be coated with a variety of coatings as needed, to reduce non-specific binding, to allow the attachment of binding ligands, for biocompatibility, for flow resistance, etc.

In a preferred embodiment, the solid support comprises ceramic materials, such as are outlined in U.S.S.N.s 09/235,081; 09/337,086; 09/464,490; 09/492,013; 09/466,325; 09/460,281; 09/460,283; 09/387,691; 09/438,600; 09/506,178; and 09/458,534; all of which are expressly incorporated by reference in their entirety. In this embodiment, the devices are made from layers of green-sheet that have been laminated and sintered together to form a substantially monolithic structure. Green-sheet is a composite material that includes inorganic particles of glass, glass-ceramic, ceramic, or mixtures thereof, dispersed in a polymer binder, and may also include additives such as plasticizers and dispersants. The green-sheet is preferably in the form of sheets that are 50 to 250 microns thick. The

ceramic particles are typically metal oxides, such as aluminum oxide or zirconium oxide. An example of such a green-sheet that includes glass-ceramic particles is "AX951" that is sold by E.I. Du Pont de Nemours and Company. An example of a green-sheet that includes aluminum oxide particles is "Ferro Alumina" that is sold by Ferro Corp. The composition of the green-sheet may also be custom formulated to meet particular applications. The green-sheet layers are laminated together and then fired to form a substantially monolithic multilayered structure. The manufacturing, processing, and applications of ceramic green-sheets are described generally in Richard E. Mistler, "Tape Casting: The Basic Process for Meeting the Needs of the Electronics Industry," Ceramic Bulletin, vol. 69, no. 6, pp. 1022-26 (1990), and in U.S. Patent No. 3,991,029, which are incorporated herein by reference.

The method for fabricating devices (such as those depicted in Figures 27-30 as devices 100 and 200) begins with providing sheets of green-sheet that are preferably 50 to 250 microns thick. The sheets of green-sheet are cut to the desired size, typically 6 inches by 6 inches for conventional processing, although smaller or larger devices may be used as needed. Each green-sheet layer may then be textured using various techniques to form desired structures, such as vias, channels, or cavities, in the finished multilayered structure.

Various techniques may be used to texture a green-sheet layer. For example, portions of a green-sheet layer may be punched out to form vias or channels. This operation may be accomplished using conventional multilayer ceramic punches, such as the Pacific Trinetics Corp. Model APS-8718 Automated Punch System. Instead of punching out part of the material, features, such as channels and wells may be embossed into the surface of the green-sheet by pressing the green-sheet against an embossing plate that has a negative image of the desired structure. Texturing may also be accomplished by laser tooling with a laser via system, such as the Pacific Trinetics LVS-3012.

Next, a wide variety of materials may be applied, preferably in the form of thick-film pastes, to each textured green-sheet layer. For example, electrically conductive pathways may be provided by depositing metal-containing thick-film pastes onto the green-sheet layers. Thick-film pastes typically include the desired material, which may be either a metal or a dielectric, in the form of a powder dispersed in an organic vehicle, and the pastes are designed to have the viscosity appropriate for the desired deposition technique, such as screen-printing. The organic vehicle may include resins, solvents, surfactants, and flow-control agents. The thick-film paste may also include a small amount of a flux, such as a glass frit, to facilitate sintering. Thick-film technology is further described in J.D. Provance, "Performance Review of Thick Film Materials," *Insulation/Circuits* (April, 1977) and in Morton L. Topfer, *Thick Film Microelectronics, Fabrication, Design, and Applications* (1977), pp. 41-59, which are incorporated herein by reference.

The porosity of the resulting thick-film can be adjusted by adjusting the amount of organic vehicle present in the thick-film paste. Specifically, the porosity of the thick-film can be increased by

increased the percentage of organic vehicle in the thick-film paste. Similarly, the porosity of a green-sheet layer can be increased by increasing the proportion of organic binder. Another way of increasing porosity in thick-films and green-sheet layers is to disperse within the organic vehicle, or the organic binder, another organic phase that is not soluble in the organic vehicle. Polymer microspheres can be used advantageously for this purpose.

To add electrically conductive pathways, the thick film pastes typically include metal particles, such as silver, platinum, palladium, gold, copper, tungsten, nickel, tin, or alloys thereof. Silver pastes are preferred. Examples of suitable silver pastes are silver conductor composition numbers 7025 and 7713 sold by E.I. Du Pont de Nemours and Company.

The thick-film pastes are preferably applied to a green-sheet layer by screen-printing. In the screen-printing process, the thick-film paste is forced through a patterned silk screen so as to be deposited onto the green-sheet layer in a corresponding pattern. Typically, the silk screen pattern is created photographically by exposure to a mask. In this way, conductive traces may be applied to a surface of a green-sheet layer. Vias present in the green-sheet layer may also be filled with thick-film pastes. If filled with thick-filled pastes containing electrically conductive materials, the vias can serve to provide electrical connections between layers.

After the desired structures are formed in each layer of green-sheet, preferably a layer of adhesive is applied to either surface of the green-sheet. Preferably, the adhesive is a room-temperature adhesive. Such room-temperature adhesives have glass transition temperatures below room temperature, *i.e.*, below about 20° C, so that they can bind substrates together at room temperature. Moreover, rather than undergoing a chemical change or chemically reacting with or dissolving components of the substrates, such room-temperature adhesives bind substrates together by penetrating into the surfaces of the substrates. Sometimes such room-temperature adhesives are referred to as "pressure-sensitive adhesives." Suitable room-temperature adhesives are typically supplied as water-based emulsions and are available from Rohm and Haas, Inc. and from Air Products, Inc. For example, a material sold by Air Products, Inc. as "Flexcryl 1653" has been found to work well.

The room-temperature adhesive may be applied to the green-sheet by conventional coating techniques. To facilitate coating, it is often desirable to dilute the supplied pressure-sensitive adhesive in water, depending on the coating technique used and on the viscosity and solids loading of the starting material. After coating, the room-temperature adhesive is allowed to dry. The dried thickness of the film of room-temperature adhesive is preferably in the range of 1 to 10 microns, and the thickness should be uniform over the entire surface of the green-sheet. Film thicknesses that exceed 15 microns are undesirable. With such thick films of adhesive voiding or delamination can occur during firing, due to the large quantity of organic material that must be removed. Films that are less

than about 0.5 microns thick when dried are too thin because they provide insufficient adhesion between the layers.

From among conventional coating techniques, spin-coating and spraying are the preferred methods. If spin-coating is used, it is preferable to add 1 gram of deionized water for every 10 grams of "Flexcryl 1653." If spraying is used, a higher dilution level is preferred to facilitate ease of spraying. Additionally, when room-temperature adhesive is sprayed on, it is preferable to hold the green-sheet at an elevated temperature, e.g., about 60 to 70° C, so that the material dries nearly instantaneously as it is deposited onto the green-sheet. The instantaneous drying results in a more uniform and homogeneous film of adhesive.

After the room-temperature adhesive has been applied to the green-sheet layers, the layers are stacked together to form a multilayered green-sheet structure. Preferably, the layers are stacked in an alignment die, so as to maintain the desired registration between the structures of each layer. When an alignment die is used, alignment holes must be added to each green-sheet layer. Typically, the stacking process alone is sufficient to bind the green-sheet layers together when a room-temperature adhesive is used. In other words, little or no pressure is required to bind the layers together. However, in order to effect a more secure binding of the layers, the layers are preferably laminated together after they are stacked.

The lamination process involves the application of pressure to the stacked layers. For example, in the conventional lamination process, a uniaxial pressure of about 1000 to 1500 psi is applied to the stacked green-sheet layers that is then followed by an application of an isostatic pressure of about 3000 to 5000 psi for about 10 to 15 minutes at an elevated temperature, such as 70° C. Adhesives do not need to be applied to bind the green-sheet layers together when the conventional lamination process is used.

However, pressures less than 2500 psi are preferable in order to achieve good control over the dimensions of such structures as internal or external cavities and channels. Even lower pressures are more desirable to allow the formation of larger structures, such as cavities and channels. For example, if a lamination pressure of 2500 psi is used, the size of well-formed internal cavities and channels is typically limited to no larger than roughly 20 microns. Accordingly, pressures less than 1000 psi are more preferred, as such pressures generally enable structures having sizes greater than about 100 microns to be formed with some measure of dimensional control. Pressures of less than 300 psi are even more preferred, as such pressures typically allow structures with sizes greater than 250 microns to be formed with some degree of dimensional control. Pressures less than 100 psi, which are referred to herein as "near-zero pressures," are most preferred, because at such pressures few limits exist on the size of internal and external cavities and channels that can be formed in the multilayered structure.

The pressure is preferably applied in the lamination process by means of a uniaxial press.

Alternatively, pressures less than about 100 psi may be applied by hand.

As with semiconductor device fabrication, many devices may be present on each sheet.

Accordingly, after lamination the multilayered structure may be diced using conventional green-sheet dicing or sawing apparatus to separate the individual devices. The high level of peel and shear resistance provided by the room-temperature adhesive results in the occurrence of very little edge delamination during the dicing process. If some layers become separated around the edges after dicing, the layers may be easily re-laminated by applying pressure to the affected edges by hand, without adversely affecting the rest of the device.

The final processing step is firing to convert the laminated multilayered green-sheet structure from its "green" state to form the finished, substantially monolithic, multilayered structure. The firing process occurs in two important stages as the temperature is raised. The first important stage is the binder burnout stage that occurs in the temperature range of about 250 to 500° C, during which the other organic materials, such as the binder in the green-sheet layers and the organic components in any applied thick-film pastes, are removed from the structure.

In the next important stage, the sintering stage, which occurs at a higher temperature, the inorganic particles sinter together so that the multilayered structure is densified and becomes substantially monolithic. The sintering temperature used depends on the nature of the inorganic particles present in the green-sheet. For many types of ceramics, appropriate sintering temperatures range from about 950 to about 1600° C, depending on the material. For example, for green-sheet containing aluminum oxide, sintering temperatures between 1400 and 1600° C are typical. Other ceramic materials, such as silicon nitride, aluminum nitride, and silicon carbide, require higher sintering temperatures, namely 1700 to 2200° C. For green-sheet with glass-ceramic particles, a sintering temperature in the range of 750 to 950° C is typical. Glass particles generally require sintering temperatures in the range of only about 350 to 700° C. Finally, metal particles may require sintering temperatures anywhere from 550 to 1700° C, depending on the metal.

Typically, the devices are fired for a period of about 4 hours to about 12 hours or more, depending on the material used. Generally, the firing should be of a sufficient duration so as to remove the organic materials from the structure and to completely sinter the inorganic particles. In particular, polymers are present as a binder in the green-sheet and in the room-temperature adhesive. The firing should be of sufficient temperature and duration to decompose these polymers and to allow for their removal from the multilayered structure.

Typically, the multilayered structure undergoes a reduction in volume during the firing process. During the binder burnout phase, a small volume reduction of about 0.5 to 1.5% is normally observed. At higher temperatures, during the sintering stage, a further volume reduction of about 14 to 17% is typically observed.

The volume change due to firing, on the other hand, can be controlled. In particular, to match volume changes in two materials, such as green-sheet and thick-film paste, one should match: (1) the particle sizes; and (2) the percentage of organic components, such as binders, which are removed during the firing process. Additionally, volume changes need not be matched exactly, but any mismatch will typically result in internal stresses in the device. But symmetrical processing, placing the identical material or structure on opposite sides of the device can, to some extent, compensate for shrinkage mismatched materials. Too great a mismatch in either sintering temperatures or volume changes may result in defects in or failure of some or all of the device. For example, the device may separate into its individual layers, or it may become warped or distorted.

As noted above, preferably any dissimilar materials added to the green-sheet layers are co-fired with them. Such dissimilar materials could be added as thick-film pastes or as other green-sheet layers, or added later in the fabrication process, after sintering. The benefit of co-firing is that the added materials are sintered to the green-sheet layers and become integral to the substantially monolithic microfluidic device. However, to be co-fireable, the added materials should have sintering temperatures and volume changes due to firing that are matched with those of the green-sheet layers. Sintering temperatures are largely material-dependent, so that matching sintering temperatures simply requires proper selection of materials. For example, although silver is the preferred metal for providing electrically conductive pathways, if the green-sheet layers contain alumina particles, which require a sintering temperature in the range of 1400 to 1600° C, some other metal, such as platinum, must be used due to the relatively low melting point of silver (961° C).

Alternatively, the addition of other substrates or joining of two post-sintered pieces can be done using any variety of adhesive techniques, including those outlined herein. For example, two "halves" of a device can be glued or fused together. For example, a particular detection platform, reagent mixture such as a hydrogel or biological components that are not stable at high temperature can be sandwiched in between the two halves. Alternatively, ceramic devices comprising open channels or wells can be made, additional substrates or materials placed into the devices, and then they may be sealed with other materials.

A particularly preferred substrate is glass, such as a microscope slide.

In a preferred embodiment, the solid substrate is configured for handling a single sample that may contain a plurality of target analytes. That is, a single sample is added to the device and the sample

may either be aliquoted for parallel processing for detection of the analytes or the sample may be processed serially, with individual targets being detected in a serial fashion. In addition, samples may be removed periodically or from different locations for in line sampling.

In a preferred embodiment, the solid substrate is configured for handling multiple samples, each of which may contain one or more target analytes. In general, in this embodiment, each sample is handled individually; that is, the manipulations and analyses are done in parallel, with preferably no contact or contamination between them. Alternatively, there may be some steps in common; for example, it may be desirable to process different samples separately but detect all of the target analytes on a single detection platform.

Furthermore, in some embodiments, the substrate comprises a multiplicity of arrays, particularly nucleic acid arrays, which are contained in one or a plurality of reaction volumes (e.g. bounded by the adhesive and covered by the flexible layer).

In addition, it should be understood that while most of the discussion herein is directed to the use of generally planar substrates with microchannels and wells, other geometries can be used as well. For example, two or more planar substrates can be stacked to produce a three dimensional device, that can contain microchannels flowing within one plane or between planes; similarly, wells may span two or more substrates to allow for larger sample volumes. Thus for example, both sides of a substrate can be etched to contain microchannels; see for example U.S. Patent Nos. 5,603,351 and 5,681,484, both of which are hereby incorporated by reference.

The biochip substrates of the invention have capture binding ligands attached in array formats. By "array" or "biochip" herein is meant a plurality of capture binding ligands, preferably nucleic acids, in an array format; the size of the array will depend on the composition and end use of the array. Most of the discussion herein is directed to the use of nucleic acid arrays with attached capture probes, but this is not meant to limit the scope of the invention, as other types of capture binding ligands (proteins, etc.), can be used. "Array" in this context generally refers to an ordered spatial arrangement, particularly an arrangement of immobilized biomolecules or polymeric anchoring structures. "Addressble array" refers to an array wherein the individual elements have precisely defined X and Y coordinates, so that a given element at a particular position in the array can be identified.

Nucleic acids arrays are known in the art, and can be classified in a number of ways; both ordered arrays (e.g. the ability to resolve chemistries at discrete sites), and random arrays are included. Ordered arrays include, but are not limited to, those made using photolithography techniques (Affymetrix GeneChip™), spotting techniques (Synteni and others), printing techniques (Hewlett Packard and Rosetta), three dimensional "gel pad" arrays, etc. The size of the array can vary; with arrays containing from about 2 different capture probes to many millions can be made, with very large

arrays being possible. Generally, the array will comprise from two to as many as 100,000, with from about 400 to about 1000 being the most preferred, and about 10,000 being especially preferred. Arrays can also be classifed as "addressable", which means that the individual elements of the array have precisely defined x and y coordinates, so that a given array element can be pinpointed.

The invention is advantageously used for performing assays using biochips **18**. Biochips, as used in the art, encompass substrates containing arrays or microarrays, preferably ordered arrays and most preferably ordered, addressable arrays, of biological molecules that comprise one member of a biological binding pair. Typically, such arrays are oligonucleotide arrays comprising a nucleotide sequence that is complementary to at least one sequence expected to be present in a biological sample. Alternatively, peptides or other small molecules can be arrayed in such biochips for performing immunological analysis (wherein the arrayed molecules are antigens) or assaying biological receptors (wherein the arrayed molecules are ligands, agonists or antagonists of said receptors). Thus, while "probes" generally refer to nucleic acids that are substantially complementary to target nucleic acids, "probe" and "biomolecular probe" can also refer to a biomolecule used to detect another biomolecule, e.g. its binding partner.

One useful feature of biochips is the manner in which the arrayed biomolecules are attached to the surface of the biochip. Conventionally such procedures involve multiple reaction steps, often requiring chemical modification of the solid support itself. Even in embodiments comprising absorption matrices, such as hydrogels, present on a solid support, chemical modification of the gel polymer is necessary to provide a chemical functionality capable forming a covalent bond with the biomolecule. The efficiency of the attachment chemistry and strength of the chemical bonds formed are critical to the fabrication and ultimate performance of the microarray.

Polymeric hydrogels and gel pads are used as binding layers to adhere to surfaces biological molecules including, but not limited to, proteins, peptides, oligonucleotides, polynucleotides, and larger nucleic acid fragments. The oligonucleotide probes may be bound to the surface of a continuous layer of the hydrogel, or to an array of gel pads. The gel pads comprising biochips for use with the apparatus of the present invention are conveniently produced as thin sheets or slabs, typically by depositing a solution of acrylamide monomer, a crosslinker such methylene bisacrylamide, and a catalyst such as N, N, N', N' - tetramethylethylendiamine (TEMED) and an initiator such as ammonium persulfate for chemical polymerization, or 2,2-dimethoxy-2-phenyl-acetophone (DMPAP) for photopolymerization, in between two glass surfaces (e.g., glass plates or microscope slides) using a spacer to obtain the desired thickness of the polymeric gel. Generally, the acrylamide monomer and crosslinker are prepared in one solution of about 4-5% acrylamide (having an acrylamide/ bisacrylamide ratio of 19/1) in water/glycerol, with a nominal amount of initiator added. The solution is polymerized and crosslinked either by ultraviolet (UV) radiation (e.g., 254 nm for at least about 15 minutes, or other appropriate UV conditions, collectively termed "photopolymerization"), or by thermal

initiation at elevated temperature (e.g., typically at about 40° C). Following polymerization and crosslinking, the top glass slide is removed from the surface to uncover the gel. The pore size (and hence the "sieving properties") of the gel is controlled by changing the amount of crosslinker and the percent solids in the monomer solution. The pore size also can be controlled by changing the polymerization temperature.

In the fabrication of polyacrylamide embodiments of the polymeric hydrogel arrays of the invention (i.e., patterned gels) used as binding layers for biological molecules, the acrylamide solution typically is imaged through a mask during the UV polymerization/crosslinking step. The top glass slide is removed after polymerization, and the unpolymerized monomer is washed away (developed) with water, leaving a fine feature pattern of polyacrylamide hydrogel, which is used to produce the crosslinked polyacrylamide hydrogel pads. Further, in an application of lithographic techniques known in the semiconductor industry, light can be applied to discrete locations on the surface of a polyacrylamide hydrogel to activate these specified regions for the attachment of an oligonucleotide, an antibody, an antigen, a hormone, hormone receptor, a ligand or a polysaccharide on the surface (e.g., a polyacrylamide hydrogel surface) of a solid support (*see, for example,* International Application, Publication No. WO 91/07087, incorporated by reference).

For hydrogel-based arrays using polyacrylamide, biomolecules (such as oligonucleotides) are covalently attached by forming an amide, ester or disulfide bond between the biomolecule and a derivatized polymer comprising the cognate chemical group. Covalent attachment of the biomolecule to the polymer is usually performed after polymerization and chemical cross-linking of the polymer is completed.

Alternatively, oligonucleotides bearing 5'-terminal acrylamide modifications can be used that efficiently copolymerize with acrylamide monomers to form DNA-containing polyacrylamide copolymers (Rehman *et al.*, 1999, *Nucleic Acids Research* 27: 649-655). Using this approach, stable probe-containing layers can be fabricated on supports (e.g., microtiter plates and silanized glass) having exposed acrylic groups. This approach has made available the commercially marketed "Acrydite™" capture probes (available from Mosaic Technologies, Boston, MA). The Acrydite moiety is a phosporamidite that contains an ethylene group capable of free-radical copolymerization with acrylamide, and which can be used in standard DNA synthesizers to introduce copolymerizable groups at the 5' terminus of any oligonucleotide probe.

With reference to the illustration provided in Figure 1, the invention provides a hybridization chamber **10** comprising a biochip, which comprises a substrate **11** having a first surface **12** and a second surface **13** opposite thereto, and a flexible layer **16** affixed to the first substrate surface **12** by an adhesive layer **15**. On the first surface **12** is an area **14** bounded by adhesive layer **15** an completely covered by flexible layer **16**. Flexible layer **16**, adhesive layer **15**, and first substrate surface **12** further

define a reaction volume **25** (also sometimes referred to herein as a reaction chamber). The ratio of volume **25** to area **14** is preferably from about 0.025 mL/mm^2 to about 0.25 mL/mm^2, more preferably from about 0.1 mL/mm^2 to about 0.25 mL/mm^2, and most preferably from about 0.1 mL/mm^2 to about 0.2 mL/mm^2.

While the present invention includes reaction volumes defined by the substrate, the adhesive and the flexible layer, as will be appreciated by those in the art, there are a variety of ways that the reaction volume can be formed. For example, rather than have an adhesive (in form of a gasket, for example) serve to create the "walls" of the chamber, the substrate itself may be formed to form these walls. As will be appreciated by those in the art, a wide variety of other configurations are also possible.

As shown in Figure 3, between flexible layer **16** and first substrate surface **12** in area **14** is positioned a multiplicity of biomolecules. In a preferred embodiment, the multiplicity of biomolecules comprises an array **17** of biomolecules, which is preferably affixed to first substrate surface **12**. Array **17** preferably further comprises gel pads **22**. In an alternate preferred embodiment, array **17** is deposited on a continuous layer of polyacrylamide. Figure 2 provides an exploded cross-sectional view of a portion of array **17** illustrating the gel pads **22**. Each gel structure **22** is preferably cylindrical, most preferably having about a 113 micron diameter and about a 25 micron thickness. The distance between each site within each array **17** is most preferably about 300 microns.

An optional layer of a water-soluble compound **28** is included that is either solid or highly viscous at a first temperature, e.g. room temperature or storage temperatures, and a liquid or more viscous at a second, higher temperature. Preferred embodiments utilize compounds having a melting point of about 30 to about 60°C, more preferably of about 35 to about 50°C, and most preferably of about 35 to about 45°C is deposited in volume **25** bounded by first substrate surface **12**, flexible layer **16**, and adhesive layer **15**. Preferably, the water-soluble compound is biocompatible, does not stick to flexible layer **16**, and serves to prevent mechanical damage to gel pads **22**. This compound can comprise any number of materials, with polymers such as glycol polymers, dextrans, sugars and other carbohydrates being preferred. In a preferred embodiment, the compound is polyethylene glycol, most preferably polyethylene glycol 600. The compound **28** is deposited so that the entire volume **25**, with the exception of that portion of volume **25** occupied by array **17**, comprises compound **28**.

Array **17** can be positioned on surface **12** by providing markings, most preferably holes or pits in surface **12**, that act as fiducials or reference points on surface **12** for accurate placement of array **17**. The presence of said fiducials is particularly advantageous in embodiments comprising a multiplicity of arrays **17** in one or a multiplicity of areas **14** on surface **12**, wherein accurate placement of said arrays is required for proper spacing and orientation of the arrays in the reaction chamber.

In preferred embodiments, a first and second port **19** and **20** extend through flexible layer **16**, as

shown in Figure 7, although in some embodiments there is only a single port that serves as both the inlet and outlet port. The first port **19** serves as an input port and is positioned in flexible layer **16** so that the first opening **29** is provided within the area **14** (reaction chamber) bounded by adhesive layer **15** on first surface **12**. Second port **20** serves as an outlet port and is positioned in flexible layer **16** so that the first opening **31** opens within area **14** bounded by the adhesive layer **15** on the first surface **12**.

Input and output ports **19** and **20** are preferably shaped to accept a plastic pipette tip, most preferably a 10µL pipette tip or a 200µL pipette tip. In preferred embodiments, input and output ports **19** and **20** are generally in the shape of a truncated cone, as shown in Figure 4, wherein the end of the cone having the smaller diameter forms the first opening of each port **29** and **31**, respectively, and the end of the cone having the larger diameter forms the second opening of each port **30** and **32**, respectively. This shape creates a seal between the pipette tip and the port, enhances visibility of the port for operator accuracy and prevents protrusion of the pipette tip into volume **25**, thereby preventing potential damage to components therein, particularly the flexible, gas permeable layer **16**. In these embodiments, each port preferably has a diameter on second substrate surface **13** of from about 1.0 mm to about 2.0 mm, and a diameter on first substrate surface **12** of from about 0.3 mm to about 0.6 mm. The conical walls of ports **19** and **20** form an angle **54** with the second substrate surface **13**, which is preferably less than 90°. Most preferably, angle **54** is less than or equal to the contact angle **55** of the biological sample fluid **26**. Most preferably, angle **54** is equal to contact angle **55** such that the surface of the fluid in the port is flat. For aqueous solutions, this angle is about 60°. This geometric arrangement provides a port that tends not to leak, but instead wicks fluid into volume **25** so that the second substrate surface **13** is dry when replaceable cover **21** is applied.

The openings of ports **19** and **20** may be covered with a removable and replaceable cover **21**. In preferred embodiments, replaceable cover **21** is a stopper, a gasket, or tape, most preferably a foil tape.

In some of these embodiments, one or more first notches **70** are cut into the first adhesive layer **15** such that the first notches **70** are in direct communication with the area **14** on first substrate surface **12** bounded by the first adhesive layer **15**. Second notches **72** are cut into the flexible layer **16** in positions corresponding to the size and position of first notches **70** in adhesive layer **15**, thus forming one or more ports. In a particularly preferred embodiment, a ring of adhesive **74** is deposited around the perimeter of each second notch **72**, such that the inner perimeter of adhesive ring **74** is coextensive with the outer perimeter of second notch **72**. Preferably, first and second notches **70** and **72** are circular in shape, and have a diameter that is equal to the inner diameter of adhesive ring **74**. Preferably the inner diameter and outer diameter of adhesive ring **74** are selected to form a tight seal with the tip end of a pipette. In an alternate preferred embodiment, a second layer of adhesive **76** is deposited on the portions of flexible layer **16** not covering the area **14** on first substrate surface **12** and

not defining first and second ports **19** and **20**. In this embodiment, the apparatus further comprises a label layer **57** that is die cut in the same manner as the first adhesive layer **15** to form windows **58** that correspond in location to areas **14** on first substrate surface **12**, and which is applied to second adhesive layer **76**. In this embodiment, one or more third notches **78** are cut into second adhesive layer **76**, such that third notches **78** correspond in shape, size, and position to first and second notches **70** and **72**. Fourth notches **80**, having a shape and position corresponding to first, second and third notches **70, 72** and **78**, are cut into label layer **57**. The diameter of fourth notches **80** is preferably greater than the diameter of first, second and third notches **70, 72** and **78**, such that after the apparatus is assembled a portion of second adhesive layer **76** is exposed by fourth notch **80**. Preferably the exposed portion of second adhesive layer **76** corresponds to the shape and size of a pipette tip.

In alternative embodiments of the apparatus, first and second ports **19** and **20** extend through substrate **11**, rather than through flexible layer **16**. Illustrative embodiments are described in co-owned and co-pending U.S. Application No. 09/464,490, incorporated by reference herein. In preferred embodiments of the apparatus, area **14** on first substrate surface **12** is square or rectangular with two rounded edges at diagonally opposite corners of are **14** and two 90 degree angles at the remaining two diagonally opposite corners of area **14**. Preferably, when first and second ports **19** and **20** extend through flexible layer **16**, first notches **70** in first adhesive layer **15** are cut at the sharp edges of area **14**, as shown in Figure 7. These embodiments are particularly preferred as they comprise geometries that eliminate corners and therefore are useful in the prevention of bubble formation in area **14**.

Substrate **11** is fabricated from any solid supporting substance, including but not limited to plastics, metals, ceramics, and glasses. Most preferably, substrate **11** is made from silicon or glass (for accuracy and stiffness), molded plastics (which reduce cost of manufacture and thermal inertia), or ceramics (for the incorporation of microfluidic elements including integrated heating elements). Most preferably, the substrate is glass.

Adhesive layer **15** is prepared using an adhesive suitable for forming a water-tight bond between substrate **11** and flexible layer **16**, including, but not limited to, high temperature acrylics, rubber-based adhesives, and silicone-based adhesives. The shape of adhesive layer **15** is configured to contain array **17**. Adhesive layer **15** can be deposited on first substrate surface **12** in a pattern to produce area **14** in any desired shape, and is most preferably deposited to define an ellipsoid area **14**. Adhesive layer **15** can be deposited using inkjet printing or offset printing methods, or by die cutting the desired shapes from a sheet of adhesive material. In addition, a substantial portion of first surface **12** can be covered with adhesive and portions of the substrate that are not desired to retain adhesive properties can be hardened preferentially, for example, by ultraviolet curing. In these embodiments,

portions retaining adhesive properties can be defined using a mask and thereby retain adhesive properties necessary to affix flexible layer **16** to surface **12**. In embodiments using the die cut adhesive material, the adhesive material is preferably a doublesided adhesive tape, and more preferably a double sided adhesive tape having no carrier. Adhesive layer **15** is most preferably set down in a layer between 1 and 100 µm thick, more preferably between 25 and 50 µm thick, and most preferably about 50 µm thick.

Flexible layer **16** is made of any flexible solid substance, including but not limited to plastics, including polypropylene, polyethylene, and polyvinylidene chloride (sold commercially as Saran® wrap) plastics, rubbers, including silicone rubbers, high temperature polyesters, and porous Teflon®. Flexible layer **16** is preferably both deformable and biocompatible and preferably has low permeability to liquids in order to prevent evaporation of water from the volume contained between the flexible layer and the substrate. That is, preferred embodiments utilized flexible layers that are selectively permeable to gas but impermeable or substantially impermeable to liquid. Flexible layer **16** also preferably is optically clear and should be able to withstand temperatures of between 50 and 95°C for a period of between 8 and 12 hours without shrinkage. Flexible layer **16** preferably covers an area of from about 5 mm^2 to about 1400 mm^2, more preferably from about 5 mm^2 to about 600 mm^2, and most preferably from about 100 mm^2 to about 600 mm^2.

In a preferred embodiment, the flexible layer is a gas permeable membrane. Most preferably, flexible, gas permeable layer **16** is selected to have physical, chemical and mechanical properties such that the surface tension of sample fluid **26** prevents passage of the sample fluid through the pores of the membrane, while allowing passage of gas molecules across the flexible, gas permeable layer. Preferably, the pore size of flexible, gas permeable layer **16** is between 0.2 and 3.0 µm, more preferably between 0.2 and 1 µm, and most preferably about 0.2 µm. Flexible, gas permeable layer **16** also preferably is translucent and should be able to withstand temperatures of between 50°C and 95°C for a period of between 8 and 12 hours without shrinkage. In a preferred embodiment, the flexible, gas permeable layer is porous Teflon®. Membranes having these characteristics are available from Pall Specialty Materials

In preferred embodiments, as shown in Figure 5, the invention further comprises a label layer **57** that is die cut in the same manner as the adhesive to form windows **58** that correspond in location to areas **14** on first substrate surface **12**. The label layer is preferably a thick film having a layer of adhesive, and most preferably is an Avery laser label. The label layer is applied to the outer surface of the flexible layer, preferably by vacuum lamination. Areas **14** are preferably visible through windows **58** in label layer **57**.

In a preferred embodiment, the invention further provides a means for facilitating diffusion across the flexible, gas permeable layer; this is referred to herein as a "gas diffusion accelerator". The gas

diffusion accelerator is used to increase the rate of diffusion of gas bubbles from the reaction chamber across the flexible layer, as compared to the diffusion rate in the absence of the accelerator. The gas diffusion accelerator can take on a variety of configurations, but is preferably removably affixed to the flexible, gas permeable layer, or the label layer when present, in order to remove gas bubbles from the reaction chambers. The gas diffusion accelerator creates a pressure gradient or concentration gradient across flexible, gas permeable layer **16**, thereby increasing the rate of diffusion of gas molecules from the sample fluid **26** contained in volume **25** across flexible, gas permeable layer **26**. A preferred embodiment of the gas diffusion accelerator , wherein the gas diffusion accelerator creates a pressure gradient across flexible, gas permeable layer **16**, is shown in Figure 14. In this embodiment, a vacuum source **70** is removably affixed to flexible, gas permeable layer **16**. In preferred embodiments, vacuum source **70** comprises a vacuum pump **71**, a chamber seal **72** that completely surrounds area **14** and is removably affixed to flexible, gas permeable layer **16**, and a length of plastic tubing **73** connecting vacuum pump **71** to reducer **72**. The chamber seal may be a suction cup, a reducer, or any other structure having similar chemical and mechanical properties. Most preferably, the plastic tubing is polyurethane tubing. Most preferably the chamber seal is made of polyvinylidene fluoride (sold under the name Kynar® by Elf Atochem North America). Diffusion-facilitating means that create a concentration gradient across the membrane are also preferred. Concentration gradients are created, for example, by providing a flow of inert gas across flexible, gas permeable layer **16**, wherein the molecules of the inert gas are too large to pass through flexible, gas permeable layer **16**, while the gas contained in volume **25** passes freely through flexible, gas permeable layer **16**. Those skilled in the art will be able to select the characteristics of flexible, gas permeable layer **16** and gas diffusion accelerators that are appropriate for their selected sample fluid **26**.

Array **17** contained in area **14** on first substrate surface **12** is optionally covered with a water-soluble compound **28**, which protects and seals the biochip prior to use and prevents degradation or other damage to the array. Any water-soluble compound **28** having a melting point of about 30°C to about 60°C, more preferably of about 35°C to about 50°C, and most preferably of about 35°C to about 45°C is advantageously used in filling volume **25** between array **17** and flexible layer **16**. Preferably, the compound is polyethylene glycol, most preferably polyethylene glycol 600. It is a particularly preferred feature of hybridization chamber **10** of the invention that water-soluble compound **28** fills the entirety of the volume **25** and more preferably also fills at least a portion of input port **19**. This prevents formation of air bubbles in volume **25** because compound **28** is first melted, then carefully mixed with the sample fluid **26** within volume **25** using a roller **40** without producing air bubbles in hybridization fluid **26**. The lack of air bubbles in reaction volume **25** enhances efficiency of the biological binding reaction by ensuring that interactions, such as hybridization, between the target analytes and the probes are capable of proceeding without interference from such air bubbles. In addition, it minimizes artifactual signals detected by a scanner **36** or a light pipe **37**.

Ports and holes can be produced in substrate **11** by diamond drilling in glass embodiments of substrate **11** or by stamping or molding in plastic embodiments thereof, or using ceramics formulation technology outlined herein. This facilitates standardization of the hybridization chamber dimensions, for example, by producing substrates where the first and second ports **19** and **20** are produced in a single operation. Both the substrate **11** and the removable cover **21** can be set down as strips or large sheets, and can be rolled to avoid trapping air bubbles. Flexible layer **16** can be applied by vacuum lamination to avoid trapping air, or can be deposited by spinning or flowing liquid plastic over substrate **11** after treatment with adhesive **15** and water-soluble compound **28**, followed by curing the flexible layer in place. Individual hybridization chambers **10** can be produced in stacks using, for example, a diamond saw as shown in Figure 6.

Figure 6 illustrates a preferred arrangement for manufacturing hybridization chamber **10**, wherein alternating layers of flexible layer **16**, adhesive layer **15**, uncut substrate **11**, and removable cover **21** are laid down, and hybridization chambers are produced by cutting the stacked layers, for example, with a diamond saw or any appropriate manufacturing tool. The sealed volumes **25** protect the arrays **17** from debris produced during the cutting process.

Alternative embodiments of the hybridization chamber **10** of the invention encompass a multiplicity of arrays **17** confined in a multiplicity of areas **14** underneath flexible layer **16**, each area comprising an array **17** and being supplied with first port **19** and, optionally, second port **20**. In these embodiments, adhesive layer **15** is deposited on first substrate surface **12** in a pattern that defines each of areas **14**, and flexible layer **16** is applied to adhesive layer **15** to encompass areas **14** on said surface.

In certain embodiments of the invention, hybridization chamber **10** is produced containing array **17** or a multiplicity of arrays **17** as disclosed herein, wherein the chamber is provided ready-to-use by the addition of hybridization fluid **26** comprising one or a multiplicity of target molecules. In alternative embodiments, hybridization chamber **10** is provided without array **17**, and allows for insertion thereof by a user. In these embodiments, at least one edge of flexible layer **16** is not adhered to first substrate surface **12** until array gas diffusion accelerator **17** is inserted.

In the use of the hybridization or reaction chamber **10** of the invention, an amount of a sample fluid **26**, most preferably comprising a biological sample containing a target nucleic acid, is added to the reaction chamber through first port **19**. Before application of the hybridization fluid **26** to the chamber, volume **25** is most preferably heated to a temperature greater than or equal to the melting temperature of water-soluble compound **28**. When melted, hybridization fluid **26** can be added to the chamber and mixed with the water-soluble compound, as shown in Figure 1B. Preferably, water-soluble compound **28** does not affect hybridization in the chamber. More preferably, the amount of compound **28** is chosen such that hybridization efficiency is improved when compound **28** is mixed with sample fluid

26.

In embodiments of the chamber comprising first port **19** but not second port **20**, the hybridization fluid is preferably introduced into the chamber after compound **28** is melted, and then the fluid is cycled into and out of the chamber using, most preferably, a pipette, until fluid **26** and compound **28** are fully mixed, and the hybridization fluid evenly distributed over the surface of array **17**, or mixed into gel pads **22** comprising certain embodiments of said arrays. Alternatively, hybridization fluid **26** is evenly distributed over the surface of array **17**, or mixed into gel pads **22** by physically manipulating flexible layer **16**, as more fully described below. In these embodiments, hybridization fluid **26** is removed after hybridization is completed, as shown in Figure 9, and array **17** is washed by the cycling a sufficient volume of a wash solution **27** into and out of the chamber, most preferably using a pipette.

In embodiments of the chamber comprising both first port **19** and second port **20**, the hybridization fluid is preferably introduced into the chamber after compound **28** is melted, and then the fluid is cycled into and out of the chamber using, most preferably, at least one pipette, until fluid **26** and compound **28** are fully mixed, and the hybridization fluid evenly distributed over the surface of array **17**, or mixed into gel pads **22** comprising certain embodiments of said biochips. Hybridization is then performed by incubating the chamber for a time and at a temperature sufficient for hybridization to be accomplished. Hybridization fluid **26** is removed after hybridization has been completed using outlet port **20**, and the biochip washed by the addition and cycling of a sufficient volume of a wash solution **27** into and out of the chamber, most preferably using a pipette. In these embodiments, the wash solution can also be continuously provided by addition through the input port and removal through the output port. In certain embodiments, the biochip containing the hybridized array is removed from the chamber for development or further manipulations as required. In preferred embodiments, the biochip containing the hybridized array is analyzed *in situ* as described below.

Prior to commencing the reaction, the reaction apparatus **10** is degassed using vacuum source **70**. Preferably a vacuum of between 13 and 27 kPa (100 to 200 torr), more preferably a vacuum of between 13 and 20 kPa (100 to 150 torr), and most preferably a vacuum of about 13 kPa (100 torr) is applied. Preferably the vacuum is applied for between 10 seconds and 2 minutes, more preferably between 10 seconds and 1 minute, most preferably between 10 seconds and 30 seconds. Vacuum source **70** is then detached from flexible, gas permeable layer **16**, and volume **25** is visually inspected for the presence of gas bubbles.

Figure 1B illustrates an advantageous embodiment of hybridization chamber **10** of the invention, further comprising a heating element **33**. Most advantageously, heating element **33** has a heating surface **34** adapted to the shape of hybridization chamber **10** to substantially cover the area **14** under flexible layer **16**. Heating element **33** is any suitable heating means, including but not limited to

resistance heaters, thermoelectric heaters, or microwave absorbing heaters.

The hybridization chamber **10** of the invention also advantageously comprises a thermocouple **35** or other temperature-sensing or measuring element to measure the temperature of hybridization fluid **26** or chamber **10**. These temperature-sensing elements advantageously are coupled with heating element **33** to control hybridization fluid **26** and wash solution **27** temperature, and can be used to calibrate other elements, such as scanning devices **36** as described below that may be sensitive to temperature.

In certain embodiments of the invention, positive hybridization is detected visually, *i.e.*, by the production of a dye or other material that reflects visible light at sites on biochip **18** where hybridization has occurred. In these embodiments, the dye or other material is most preferably produced enzymatically, for example, using a hybridization-specific immunological reagent such as an antibody linked to an enzyme that catalyzes the production of the dye. Visual inspection can be used to detect sites of positive hybridization. More preferably, the biochip containing the hybridized array is scanned using scanner **36** as disclosed more fully below.

Positive hybridization on biochip **18** most preferably is detected by fluorescence using labeled target molecules in a biological sample, or by including intercalating dyes in the hybridization fluid **26** that fluoresce when bound by a hybridized DNA duplex and illuminated by light at a particular wavelength. Suitable intercalating dyes include, but are not limited to, ethidium bromide, Hoechst DAPI, and Alexa Fluor dyes. Suitable fluorescence labels include, but are not limited to, fluorescein, rhodamine, propidium iodide, and Cy3 and Cy5 (Amersham), that can be incorporated into target molecules, for example, *in vitro* amplified fragments using labeled oligonucleotide primers.

Figures 10A-10C illustrate an embodiment of the invention comprising a scanner **36**, which is advantageously positioned over (or beneath) flexible layer **16** and moves from one end of area **14** to the opposite end, sequentially illuminating area **14** and array **17** positioned thereupon. Prior to analysis of the hybridized array, all fluid is removed from volume **25** such that flexible layer **16** is in contact with array **17**. Scanner **36** then excites the fluorescent dye, preferably with short wavelength light, most preferably light with a wavelength between 250 nm and 600 nm. Scanner **36** then collects the emitted light from a specific area. The amount of light emitted is then used to determine the amount of fluorescent dye present in the area, and hence the amount of labeled target.

Particular embodiments of scanners and scanning devices **36** are shown in Figures 11A through 11E. It is a particularly advantageous feature of hybridization chamber **10** that flexible layer **16** is translucent to suitable wavelengths of light, including light in the ultraviolet and visible portion of the spectrum. An additional advantageous feature of hybridization chamber **10** is that flexible layer **16**, which is very thin,

is immediately adjacent to and in contact with biochip **18** after hybridization fluid **26** or wash fluid **27** is removed from the chamber. This combination of features reduces or eliminates free surface reflections, internal reflection of illumination from the scanner, and dispersion or scattering of illuminating light, thereby optimizing the amount of incident light that illuminates array **17**. This arrangement is also more economical than in existing apparatus as it minimizes the need for highly polished, low scattering surfaces or complex or expensive lenses, and eliminates problems associated with focus and depth-of-field in more complex optical detectors.

In other embodiments, a light pipe **37** contacts the surface of flexible layer **16** that is immediately adjacent to and in contact with the surface of array **17**, as shown in Figure 11B. In these embodiments, both illuminating and emitted light are conveyed and collected by light pipe **37**. The pipe is designed to be slightly flexible so as to adapt to the contoured surface of flexible layer **16**. Light pipe **37** contacts flexible layer **16** that contacts array **17**, thereby permitting contacts free of surface reflections even under circumstances where array **17** or light pipe **37** has localized variations in height. Advantageously, light pipe **37** has a larger surface area than array **17**, so that the maximum amount of illuminating light is delivered to array **17**, and the maximum amount of emitted light from array **17** is collected by light pipe **37**. A further advantage of light pipe **37** is that it enables detection of bubbles formed in hybridization fluid **26** or wash buffer **27**, which detection can be used as a signal for roller **40** to address flexible layer **16** to remove such bubbles. Removing bubbles in hybridization fluid **26** or wash buffer **27** reduces the frequency of non-specific binding and artifactual signals detected by scanner **36**.

In additional embodiments of the invention, the area **14** defined by adhesive layer **15** further comprises a reflective layer **38** that substantially covers the entirety of the area **14** and is positioned between array **17** and the first substrate surface **12**. In preferred embodiments, reflective layer **38** comprises aluminum, gold, silver, or platinum. In these embodiments, the amount of the light signal reflected or transmitted to the light-detecting portion of scanner **36** is increased up to four-fold. In further advantageous embodiments of the invention, reflective layer **38** is a metal film resistor or an RF induction heater. In these embodiments, reflective **38** layer can heat the slide without requiring additional heating elements **33**. This is a particularly desirable feature in hand-held embodiments of the hybridization chamber **10** of the invention.

If required, a passivation later **39** can be applied on top of reflective layer **38**. Preferably, passivation layer **39** is a layer of parylene a few microns thick that is applied by evaporation. The amount of illumination required, and hence the amount of power needed to operate scanner **36** is reduced in these embodiments, which are particularly suited to battery-operated embodiments such as hand-held devices to improve useful battery life. Furthermore, passivation layer **39** reduces artifactual signals in the light emission data by obscuring objects that it covers.

Hybridization chamber **10** is preferably supplied with a roller **40** in removable contact with flexible layer **16** and that can be moved longitudinally across areas **14** on first substrate surface **12**. In preferred embodiments, the surface of roller **40** comprises a textured pattern **41**, most preferably a spiral pattern, that permits the roller to efficiently mix hybridization fluid and wash solution across area **14** and array **17**. The roller can move longitudinally across the surface of the chamber for mixing sample fluid and wash solutions as required. One advantageous arrangement of roller **40** (again, preferably a patterned roller) and hybridization chamber **10** is shown in Figure 11E. As shown in the Figure, roller **40** can be advantageously connected to a movable arm **42** that can be positioned to place roller **40** in contact with flexible layer **16** when in a first position, and can be moved to a second position in which roller **40** is not in contact with flexible layer **16**. Most preferably, movable arm **42** has a pivot point **44** and movement about said pivot point is preferably controlled by a solenoid. In addition to movement of roller **40** in contact with and away from hybridization chamber **10**, either roller **40** or hybridization chamber **10**, or both, are movable in a longitudinal direction to enable roller **40** to mix hybridization fluid **26** or wash solution **27** inside volume **25** bounded by flexible layer **16**, adhesive layer **15**, and first substrate surface **12** in area **14** containing array **17**. In embodiments comprising a multiplicity of areas **14** containing a multiplicity of arrays **17**, roller **40** is positioned to move longitudinally across each of the multiplicity of areas **14** to mix hybridization fluid **26** or wash solution **27** in each of the volumes **25** containing arrays **17**.

In additional embodiments, a sample preparation chip **45**, comprising a port **46**, as shown in Figures 12A through 12C, can be attached to hybridization chamber **10**. Most preferably, port **46** in sample preparation chip **45** is aligned with first port **19** in hybridization chamber **10** to permit efficient transfer of sample to volume **25**. Additional fiducial references can be used to accurately align hybridization chamber **10** and sample preparation chip **45**. Since access to first port **19** is through second substrate surface **13**, the array can be scanned without interference from the attached sample preparation chip. In alternative embodiments of the invention, sample preparation chip **45** may be bound to second substrate surface **13** (Figure 12B) or formed as an integral part of substrate **11** (Figure 12C).

A preferred embodiment of hybridization chamber **10** of the invention is a hand-held embodiment as shown in Figures 10A-10C, further comprising a scanner **36**. In these embodiments, hand-held device **47** comprises a base **48**, a lid **49** and a carriage **50** embodying roller **40**, scanner **36**, heating element **33** and thermocouple **35**. Carriage **50** is illustrated in Figure 11A. Device **47** comprises a compartment **51** for positioning hybridization chamber **10** in proximity to carriage **50**. Carriage **50** is provided with moving means for moving roller **40**, scanner **36** and heating element **33** relative to hybridization chamber **10** as required for operation as described above. Carriage **50** and lid **49** are arranged to permit a user to introduce and remove hybridization fluid **26** and wash solution **27** into the chamber through first port **19** and second port **20** as required. Alternatively, device **47** further comprises fluidic connections **52** to each of the first and second ports to provide for sample

introduction and array washing after hybridization of the sample thereto. Device **47** is most preferably operated by battery, although AC adapters are also advantageously encompassed by the description of the device herein. In further preferred embodiments, lid **49** further comprises a display **56** for displaying the results of the analysis.

With respect to the methods of using the devices, there are a wide variety of methods that can be used. If required, the target sequence is prepared using known techniques. For example, the sample may be treated to lyse the cells, using known lysis buffers, sonication, electroporation, etc., with purification and amplification as outlined below occurring as needed, as will be appreciated by those in the art. In addition, the reactions outlined herein may be accomplished in a variety of ways, as will be appreciated by those in the art. Components of the reaction may be added simultaneously, or sequentially, in any order, with preferred embodiments outlined below. In addition, the reaction may include a variety of other reagents which may be included in the assays. These include reagents like salts, buffers, neutral proteins, e.g. albumin, detergents, etc., which may be used to facilitate optimal hybridization and detection, and/or reduce non-specific or background interactions. Also reagents that otherwise improve the efficiency of the assay, such as protease inhibitors, nuclease inhibitors, anti-microbial agents, etc., may be used, depending on the sample preparation methods and purity of the target.

In addition, in most embodiments, double stranded target nucleic acids are denatured to render them single stranded so as to permit hybridization of the primers and other probes of the invention. A preferred embodiment utilizes a thermal step, generally by raising the temperature of the reaction to about 95°C, although pH changes and other techniques may also be used.

As outlined herein, the invention provides a number of capture probes that will hybridize to some portion, i.e. a domain, of the target sequence. Probes of the present invention are designed to be complementary to a target sequence (either the target sequence of the sample or to other probe sequences, for example for use in sandwich assays known in the art) such that hybridization of the target sequence and the probes of the present invention occurs. As outlined below, this complementarity need not be perfect; there may be any number of base pair mismatches which will interfere with hybridization between the target sequence and the single stranded nucleic acids of the present invention. However, if the number of mutations is so great that no hybridization can occur under even the least stringent of hybridization conditions, the sequence is not a complementary target sequence. Thus, by "substantially complementary" herein is meant that the probes are sufficiently complementary to the target sequences to hybridize under normal reaction conditions.

A variety of hybridization conditions may be used in the present invention, including high, moderate and low stringency conditions; see for example Maniatis et al., Molecular Cloning: A Laboratory Manual, 2d Edition, 1989, and Short Protocols in Molecular Biology, ed. Ausubel, et al, hereby

incorporated by reference. Stringent conditions are sequence-dependent and will be different in different circumstances. Longer sequences hybridize specifically at higher temperatures. An extensive guide to the hybridization of nucleic acids is found in Tijssen, Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Acid Probes, "Overview of principles of hybridization and the strategy of nucleic acid assays" (1993). Generally, stringent conditions are selected to be about 5-10°C lower than the thermal melting point (Tm) for the specific sequence at a defined ionic strength and pH. The Tm is the temperature (under defined ionic strength, pH and nucleic acid concentration) at which 50% of the probes complementary to the target hybridize to the target sequence at equilibrium (as the target sequences are present in excess, at Tm, 50% of the probes are occupied at equilibrium). Stringent conditions will be those in which the salt concentration is less than about 1.0 M sodium ion, typically about 0.01 to 1.0 M sodium ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes (e.g. 10 to 50 nucleotides) and at least about 60°C for long probes (e.g. greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of helix destabilizing agents such as formamide. The hybridization conditions may also vary when a non-ionic backbone, i.e. PNA is used, as is known in the art. In addition, cross-linking agents may be added after target binding to cross-link, i.e. covalently attach, the two strands of the hybridization complex.

Thus, the assays are generally run under stringency conditions which allows formation of the hybridization complex only in the presence of target. Stringency can be controlled by altering a step parameter that is a thermodynamic variable, including, but not limited to, temperature, formamide concentration, salt concentration, chaotropic salt concentration, pH, organic solvent concentration, etc.

These parameters may also be used to control non-specific binding, as is generally outlined in U.S. Patent No. 5,681,697. Thus it may be desirable to perform certain steps at higher stringency conditions to reduce non-specific binding.

As described herein, there are a number of possible detection techniques that can be utilized in the present invention. In a preferred embodiment, as outlined herein, optical label techniques are used. In these embodiments, a label such as an optical dye (e.g. a fluorochrome) is added to the assay complex comprising the target analyte and the capture binding ligand. In some embodiments, for example in the case of nucleic acids, the label can be added to the target, for example by incorporation during an amplification reaction such as PCR. For example, the fluorochromes or other labels such as biotin can be added to the PCR primers or to the dNTPs for enzymatic incorporation. Alternatively, intercalators can be used as described above.

Alternatively, preferred embodiments allow the use of electrical detection methods such as those outlined in U.S.S.N.s 09/458,553; 09/458,501; 09/572,187; 09/495,992; 09/344,217; WO00/31148; 09/439,889; 09/438,209; 09/344,620; 09/478,727; PCTUS00/17422; WO 98/20162; WO 98/12430;

WO 98/57158; WO 99/57317; WO 99/67425; PCT 00/19889; and WO 99/57319, all of which are expressly incorporated by reference in their entirety. These embodiments utilize arrays of microelectrodes on the substrate.

The Examples that follow are illustrative of specific embodiments of the invention and various uses thereof. They are set forth for explanatory purposes only, and are not to be taken as limiting the invention. All references cited herein are expressly incorporated by reference in their entirety.

EXAMPLE 1

ASSEMBLY OF A HYBRIDIZATION CHAMBER

The process of assembling a chamber according to the present invention is illustrated in Figure 13.

A die cutter was used to cut four ellipsoidal holes in a layer of 502FL ultra-clean laminating adhesive film (3M). A similar pattern was punched into an Avery laser label 5663 for use as a frame and label layer. Meanwhile, a sheet of polyvinylidene chloride film was stretched over a stainless steel frame and annealed for 30 minutes at 100°C. The Avery label was applied to one side of the polyvinylidine chloride film by vacuum laminating the label in a vacuum lamination press. A vacuum of 15 psi was applied for 30 seconds, and mechanical pressure of 15 psi was maintained for 1 minute after release of the vacuum. The adhesive was then applied to the opposite side of the polyvinylidene chloride film using the same process as for the label.

The adhesive coated film was then applied to a glass slide that had previously been prepared. The arrays of oligonucleotide probes and gel pads were positioned on the glass slide using standard methods. Ports were drilled into the slide using a diamond drill. A vacuum lamination press was used to affix the polyvinylidene chloride film to the slide. A vacuum of 15 psi was maintained for 1 minute, and then mechanical pressure of 15 psi was maintained for an additional minute.

The individual chambers were then filled with polyethylene glycol 600 using a 10 mL pipette tip. A layer of 3M 7350 polyester tape was then applied to the slide to seal off the ports.

EXAMPLE 2

ASSEMBLY OF A TOP-LOADING HYBRIDIZATION CHAMBER

A die cutter was used to cut four ellipsoidal holes in a layer of 502FL ultra-clean laminating adhesive film (3M). A similar pattern was punched into an Avery laser label 5663 for use as a frame and label layer. Meanwhile, a sheet of polyvinylidene chloride film was stretched over a stainless steel frame and annealed for 30 minutes at 100°C. The Avery label was applied to one side of the polyvinylidine chloride film by vacuum laminating the label in a vacuum lamination press. A vacuum of 15 psi was applied for 30 seconds, and mechanical pressure of 15 psi was maintained for 1 minute after release of the vacuum. The adhesive was then applied to the opposite side of the polyvinylidene chloride film using the same process as for the label.

The adhesive coated film was then applied to a glass slide that had previously been prepared. The arrays of oligonucleotide probes and gel pads were positioned on the glass slide using standard methods. A vacuum lamination press was used to affix the polyvinylidene chloride film to the slide. A vacuum of 15 psi was maintained for 1 minute, and then mechanical pressure of 15 psi was maintained for an additional minute.

The individual chambers were then filled with polyethylene glycol 600 using a 10 mL pipette tip. A layer of 3M 7350 polyester tape was then applied to the slide to seal off the ports.

EXAMPLE 3

REMOVING GAS BUBBLES FROM A REACTION CHAMBER

The process of assembling a chamber according to the present invention is illustrated in Figure 14.

A four reaction-chamber apparatus is manufactured using a layer of 0.2 µm porous Teflon unsupported membrane as the flexible, gas permeable layer, following the procedure provided in U.S. Application Serial No. 09/464,490, incorporated by reference herein. Each reaction chamber is filled with 75 µL of a sample fluid containing biological target molecules by injection through a 300 µL pipette tip (VWR Part No. 53510-084) using a 200 µL pipettor (Rainin P-200). Bubbles are visually detectable in the chambers after injection.

A reaction chamber is isolated by applying a Cole-Parmer Kynar 1/4" x 5/8" barbed reducer (Part No. 31513-31) directly to the frame layer and forming a seal around the chamber. A "house" vacuum source is connected to the reducer by a length of polyurethane tubing. A vacuum of 200 torr is applied for two minutes. Visual inspection of the chamber following application of the vacuum shows no gas bubbles remaining in the chamber.

The reaction apparatus is maintained at 25°C and atmospheric pressure for 8 hours until the reaction proceeds to completion. No appreciable evaporation of water from the chamber is observed.

It should be understood that the foregoing disclosure emphasizes certain specific embodiments of the invention and that all modifications or alternatives equivalent thereto are within the spirit and scope of the invention as set forth in the appended claims.

CLAIMS

We claim:

1. An apparatus for performing biological reactions comprising:
   a) a substrate having a first surface and a second surface;
   b) an array of biomolecules positioned on said first surface;
   c) a porous flexible layer affixed to said first surface by an adhesive layer to create a reaction volume;
   d) a gas diffusion accelerator; and
   e) a first port extended from said second surface to said reaction volume of said first surface.

2. An apparatus according to claim 1 wherein said biomolecules are nucleic acids.

3. An apparatus according to claim 1 or 2 wherein said substrate is glass, silicon, ceramic or plastic.

4. An apparatus according to claim 1, 2 or 3 wherein said biomolecules are nucleic acids.

5. An apparatus according to claim 1, 2, 3 or 4 wherein said biomolecules are attached to said substrate using a gel pad.

6. An apparatus according to claim 1, 2, 3, 4 or 5 wherein said reaction volume comprises a water-soluble compound that is a solid at a first temperature and a liquid at a second higher temperature.

7. An apparatus according to claim 1, 2, 3, 4, 5 or 6 wherein said porous flexible layer is porous Teflon™.

8. An apparatus according to claim 1, 2, 3, 4, 5, 6 or 7 wherein said gas diffusion accelerator comprises a vacuum source affixed to said porous flexible layer.

9. An apparatus according to claim 8 wherein said vacuum source comprises:
   a) a vacuum pump; and
   b) a chamber seal affixed to said reaction volume.

10. A method of detecting the presence of a target analyte in a sample comprising:
   a) contacting said sample with an apparatus comprising:
      i) a substrate having a first surface and a second surface;
      ii) an array of biomolecules positioned on said first surface;
      iii) a porous flexible layer affixed to said first surface by an adhesive

layer to create a reaction volume;

iv) a gas diffusion accelerator; and

v) a first port extended from said second surface to said reaction volume of said first surface;

under conditions whereby said target analyte will bind to at least one of said biomolecules;

b) utilizing said gas diffusion accelerator to remove gas bubbles; and

c) detecting the presence of said target analyte.

11. A method according to claim 10 wherein said biomolecules are nucleic acids.

FIG._1A

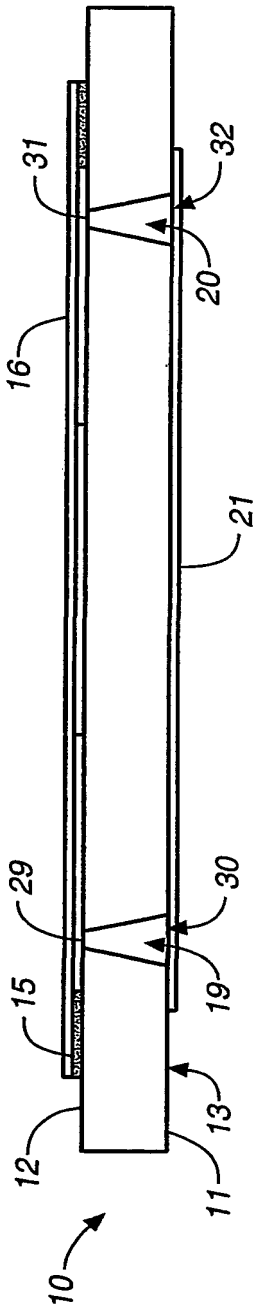


FIG._1B

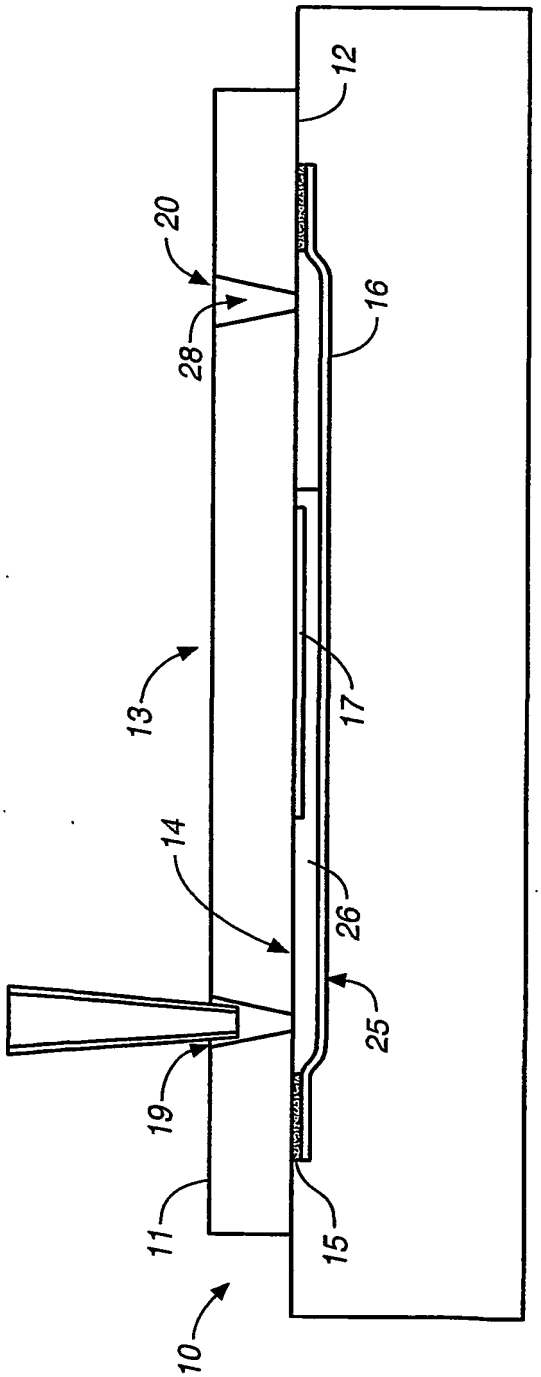
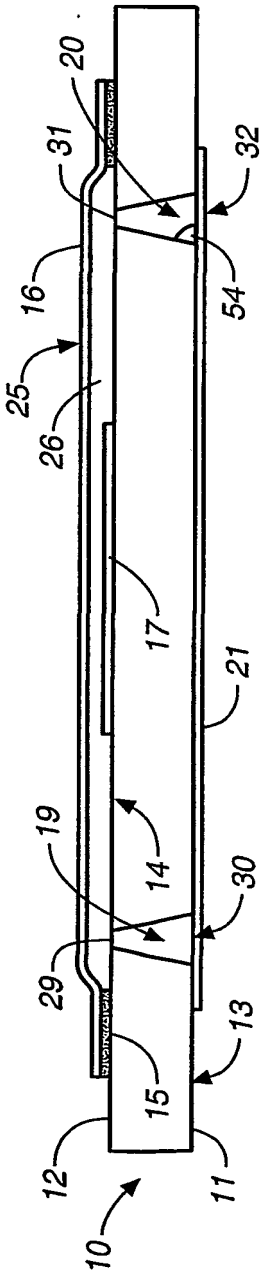


FIG._1C



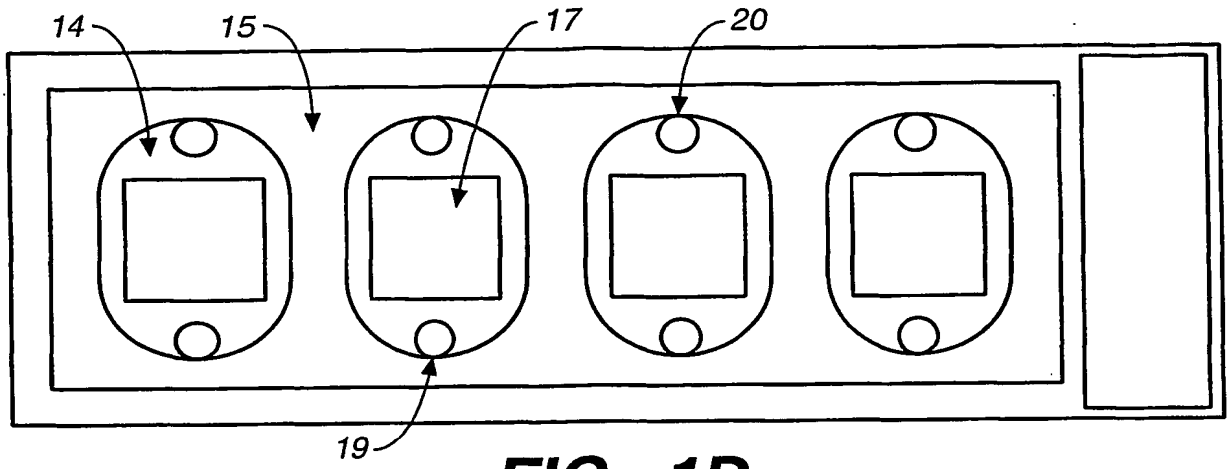


FIG._1D

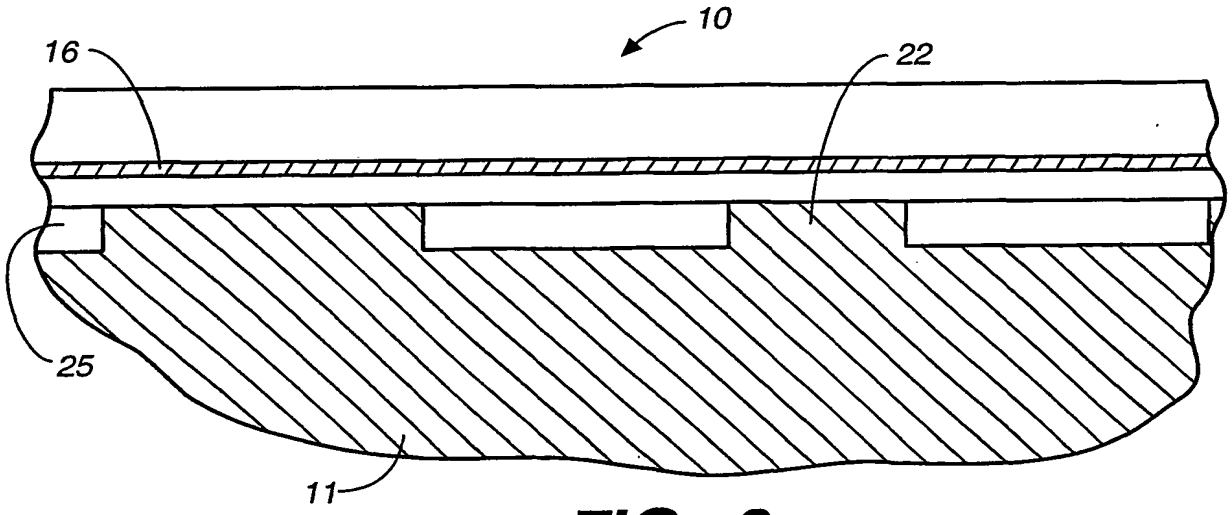


FIG._2

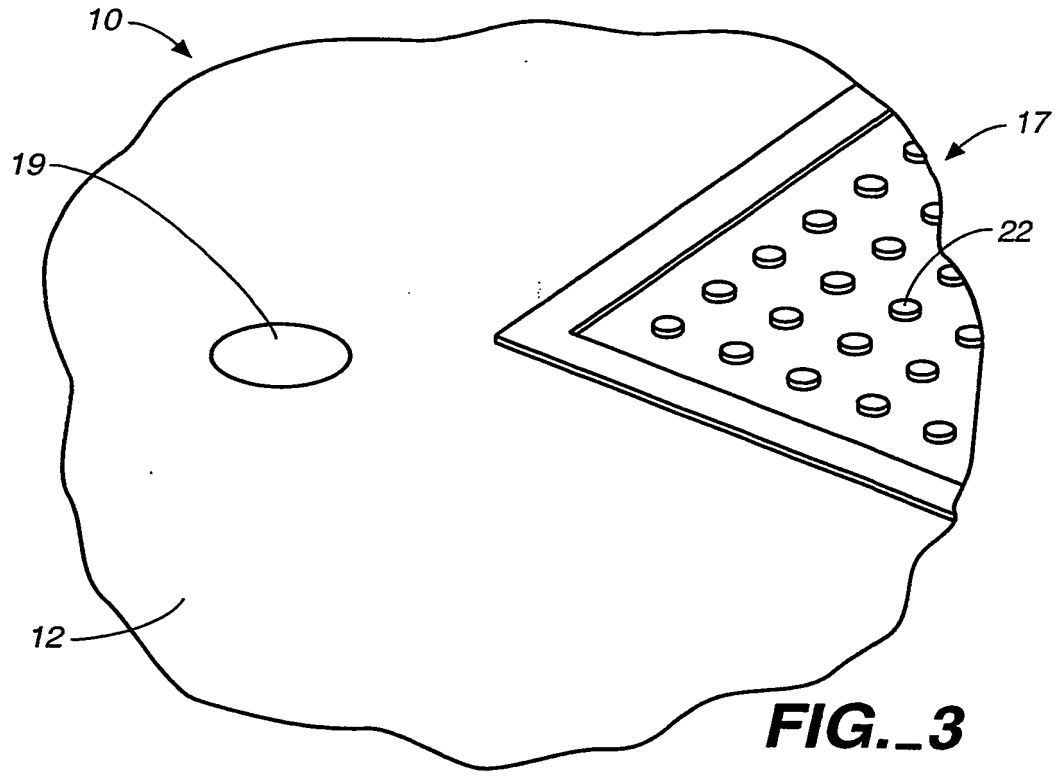


FIG._3

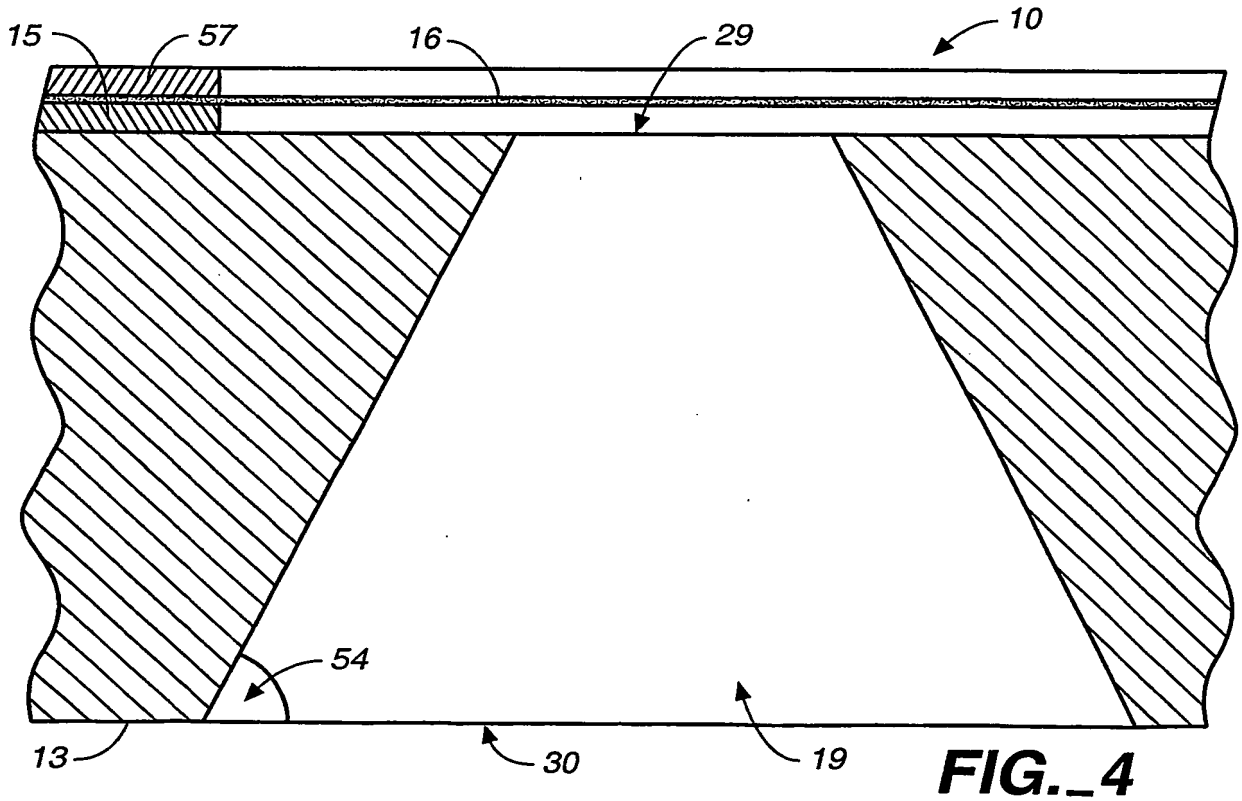


FIG._4

FIG._5

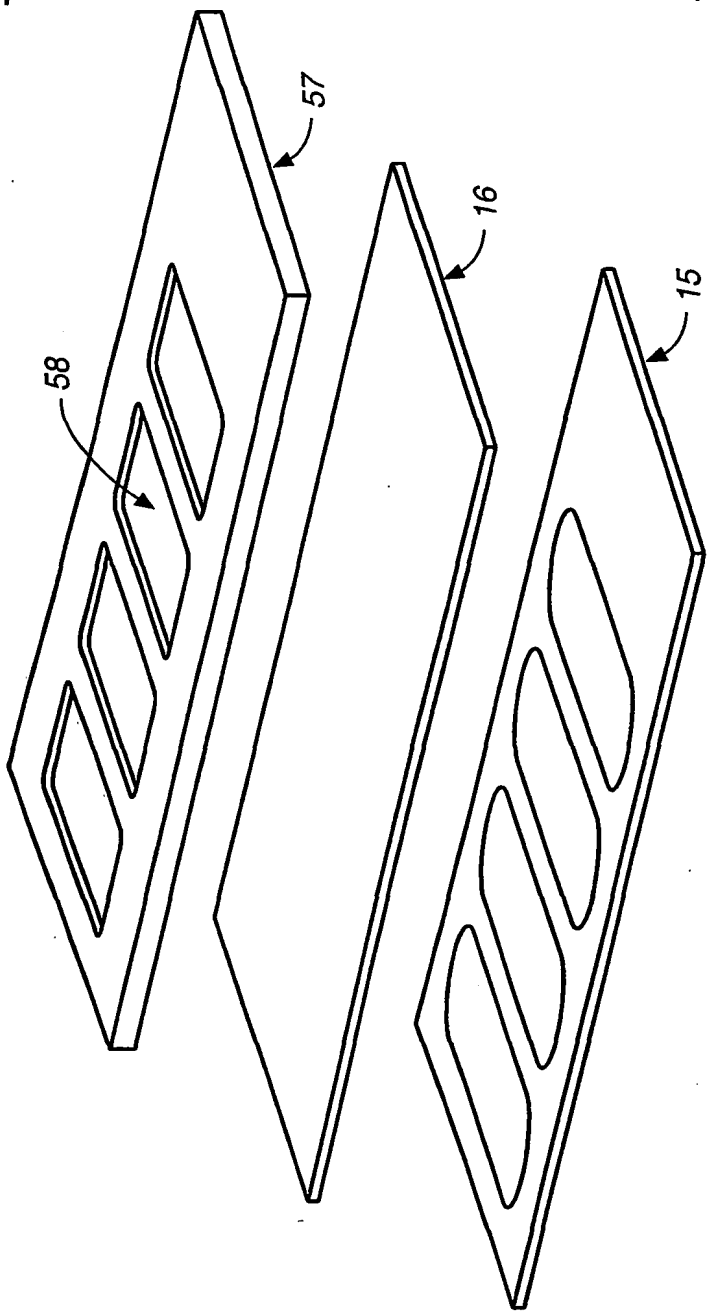
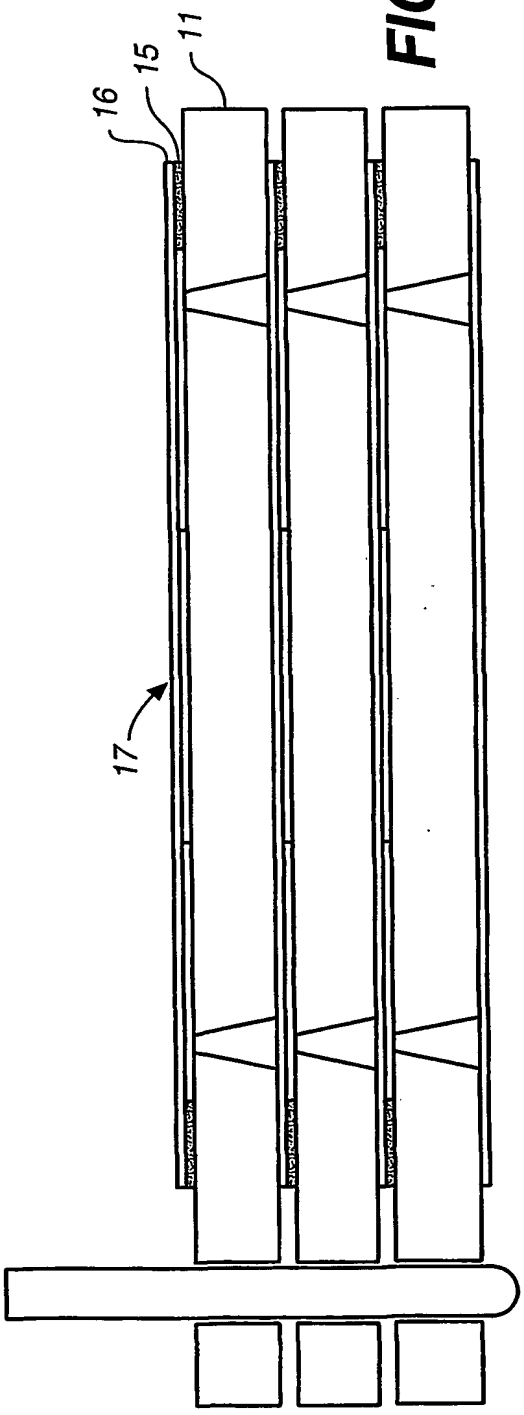
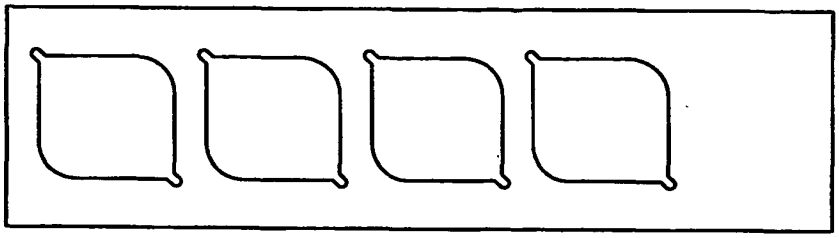


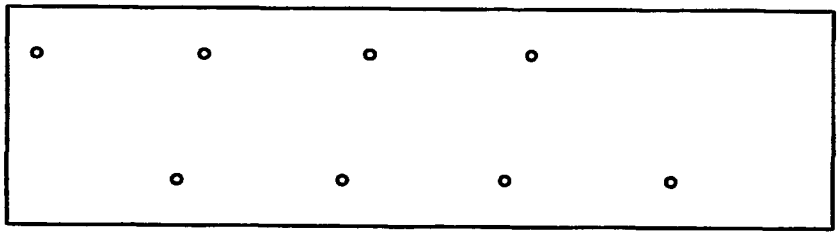
FIG._6





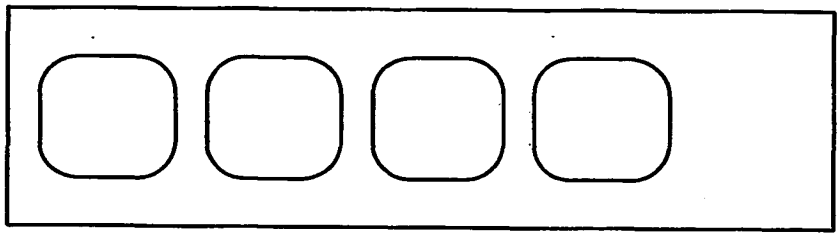
bottom adhesive layer

FIG._7A



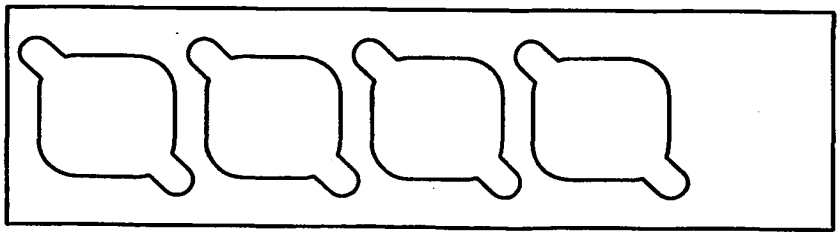
film layer

FIG._7B



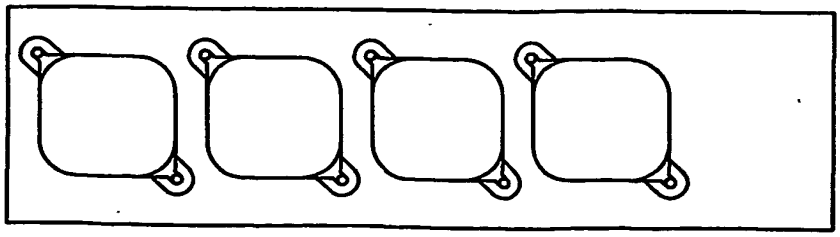
upper adhesive layer

FIG._7C



Label frame

FIG._7D



assembled label

FIG._7E

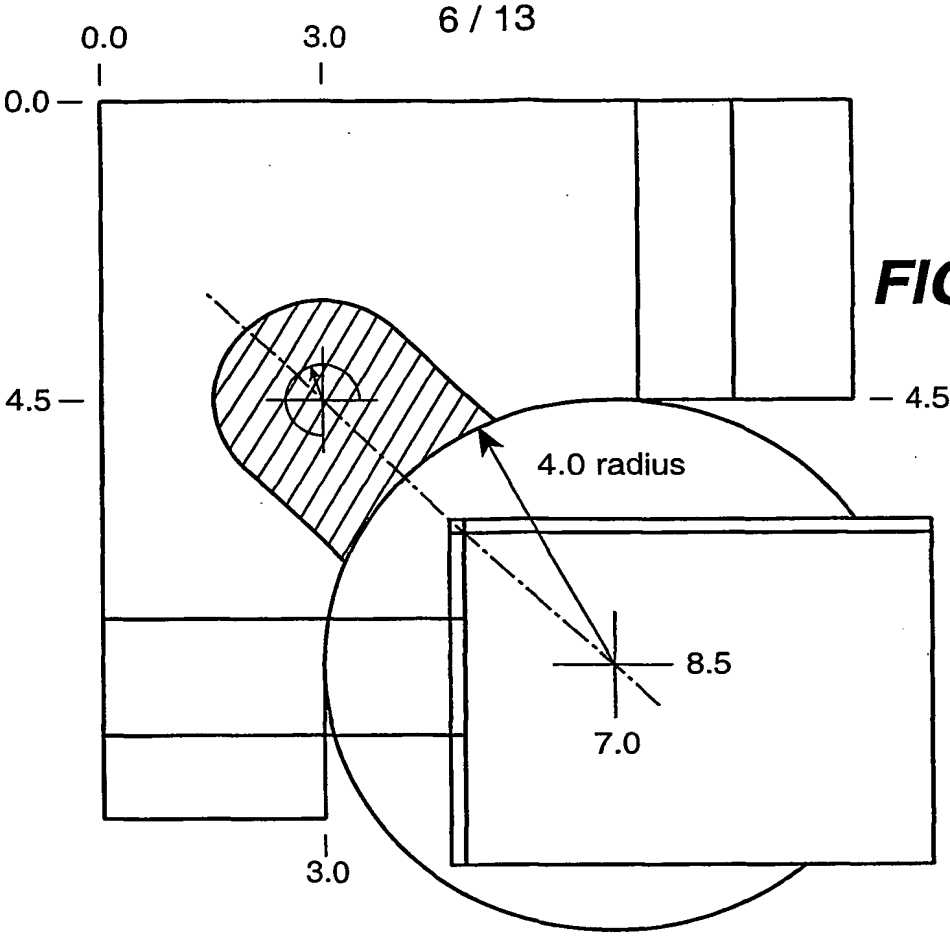


FIG._8A

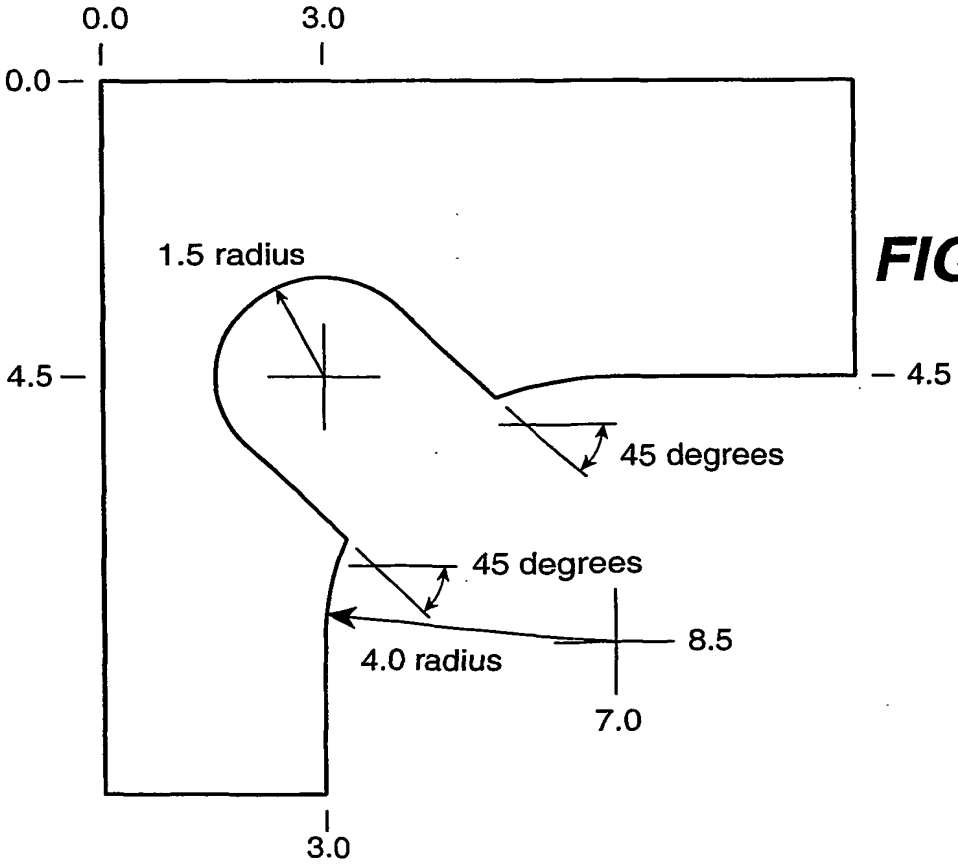


FIG._8B

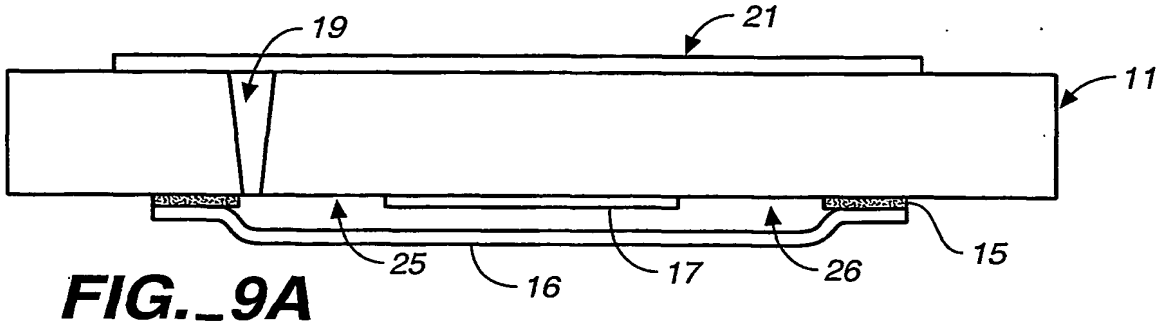


FIG._9A

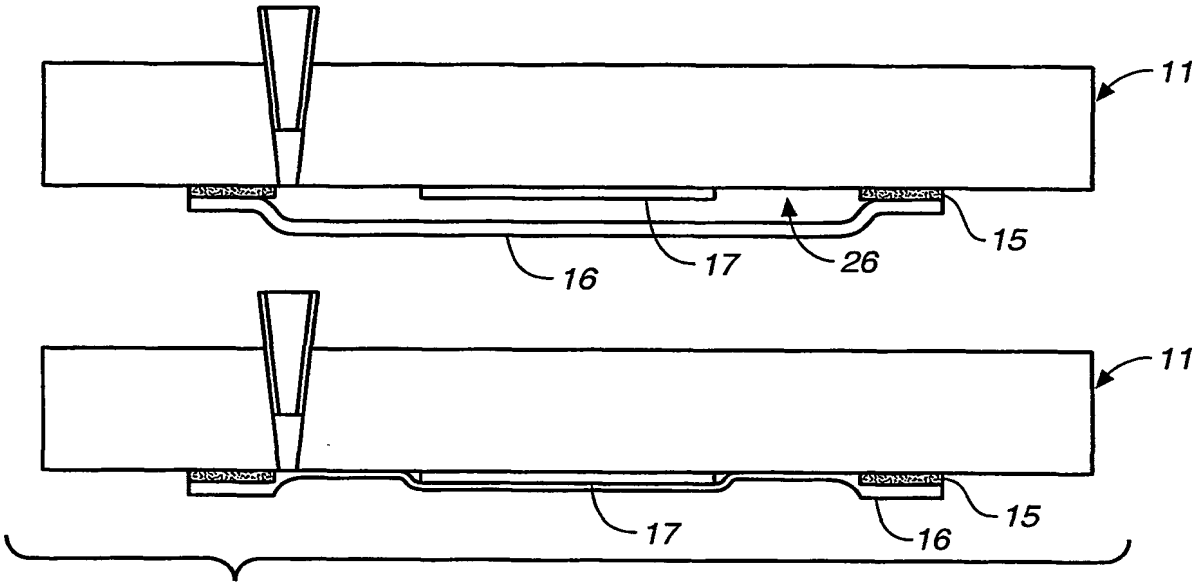


FIG._9B

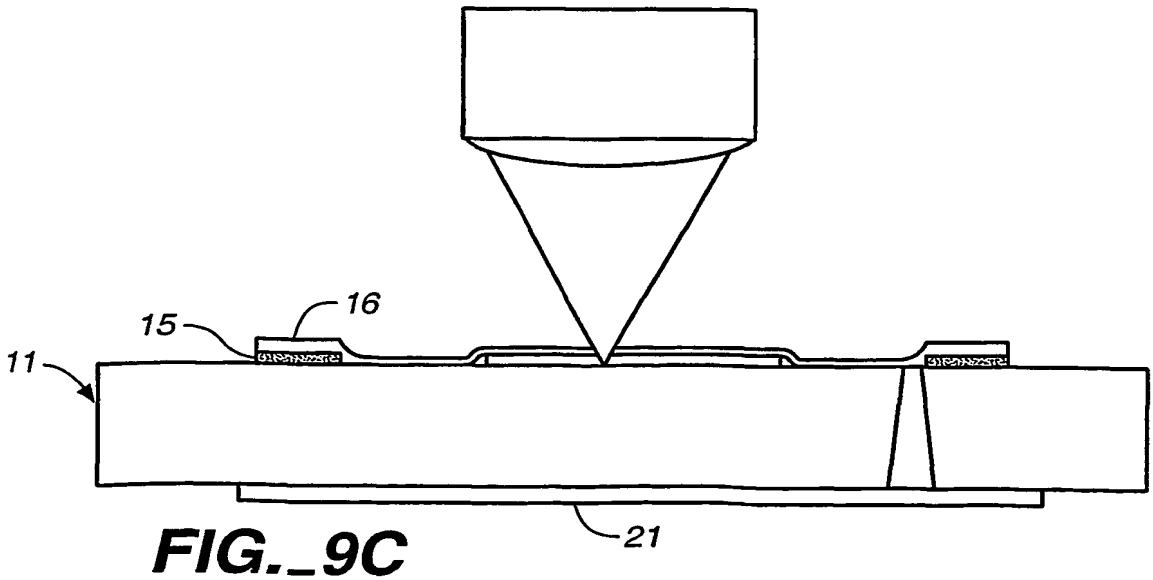


FIG._9C

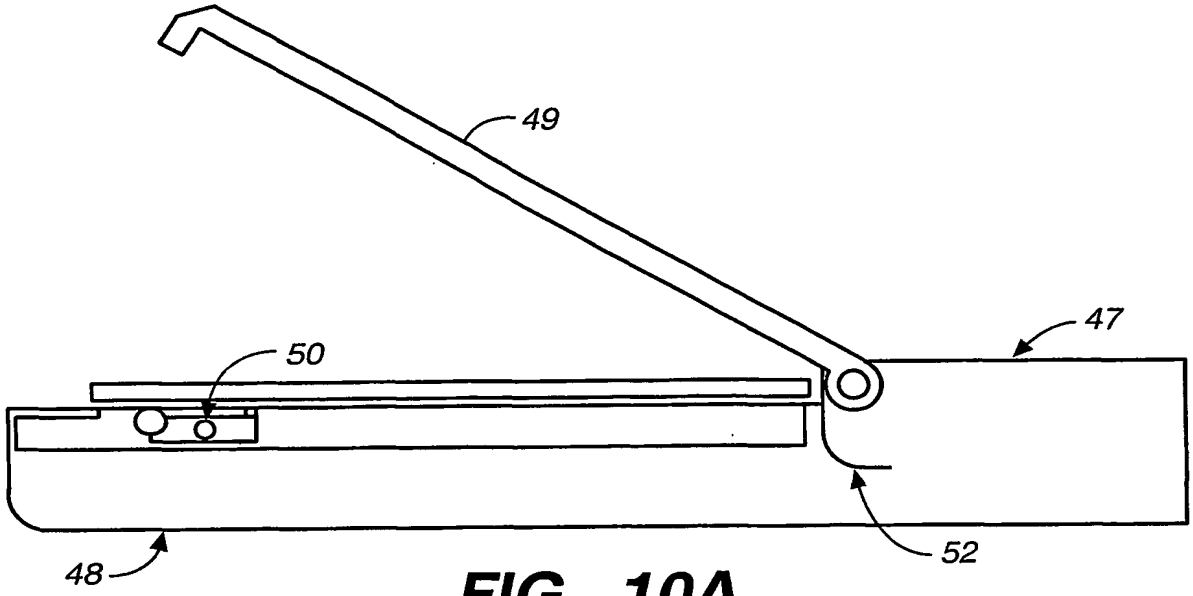


FIG._10A

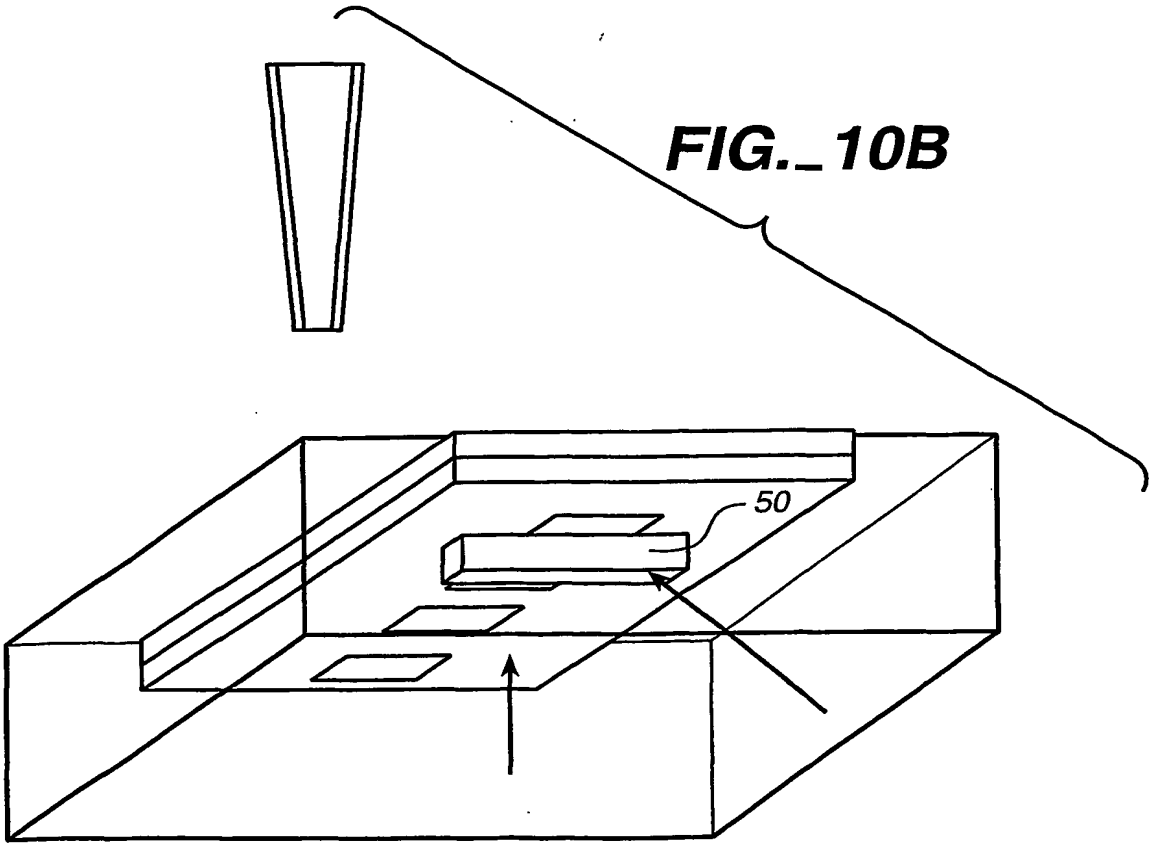


FIG._10B

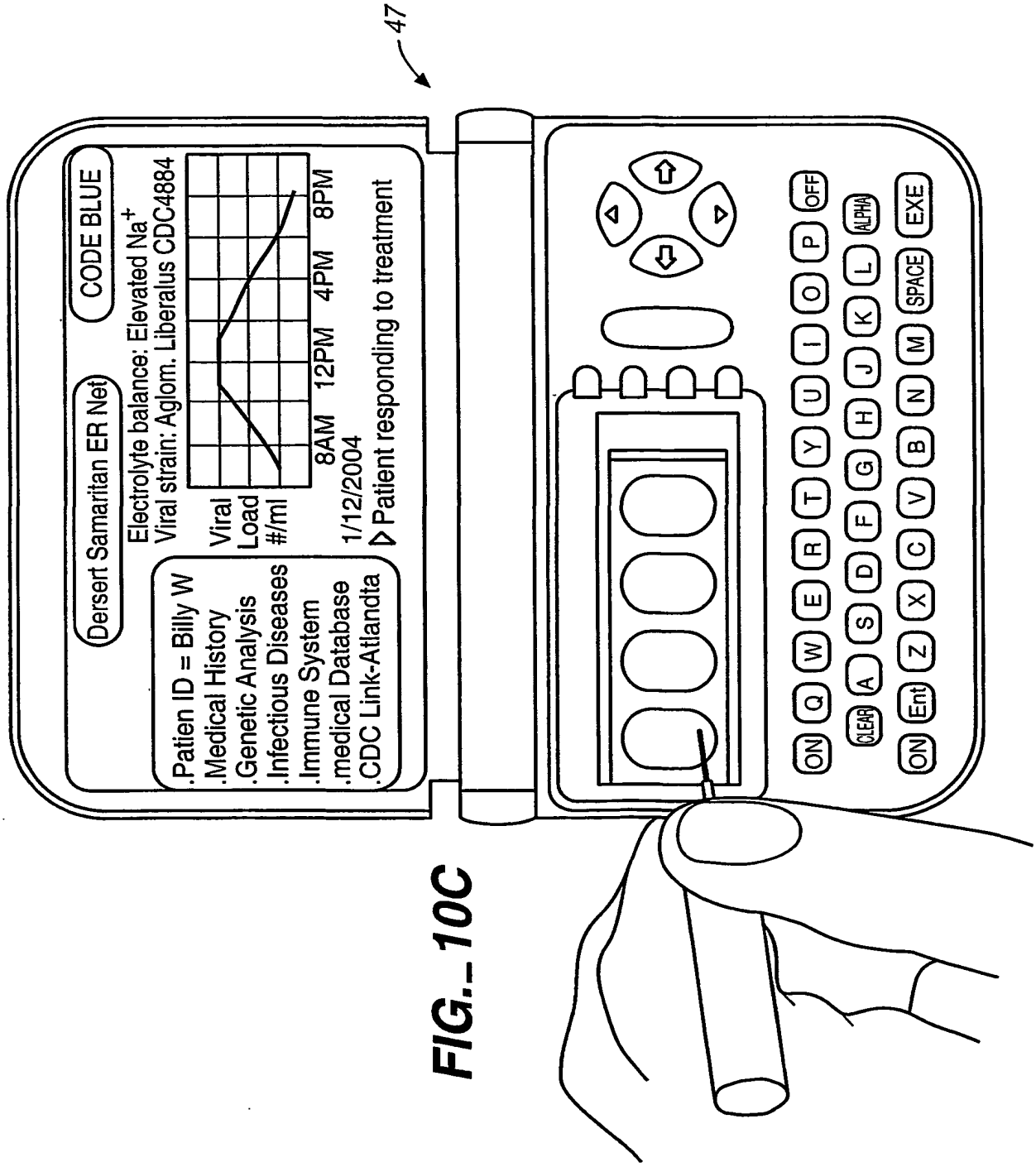


FIG._10C

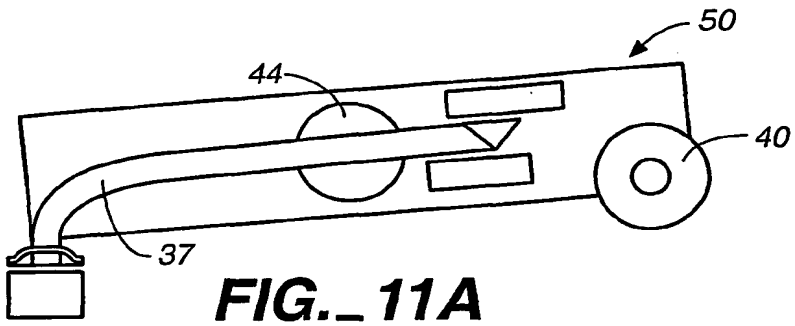


FIG._11A

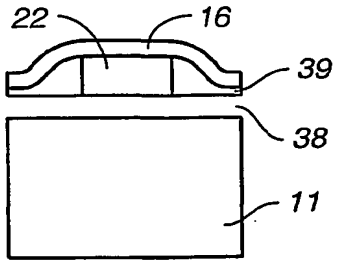


FIG._11C

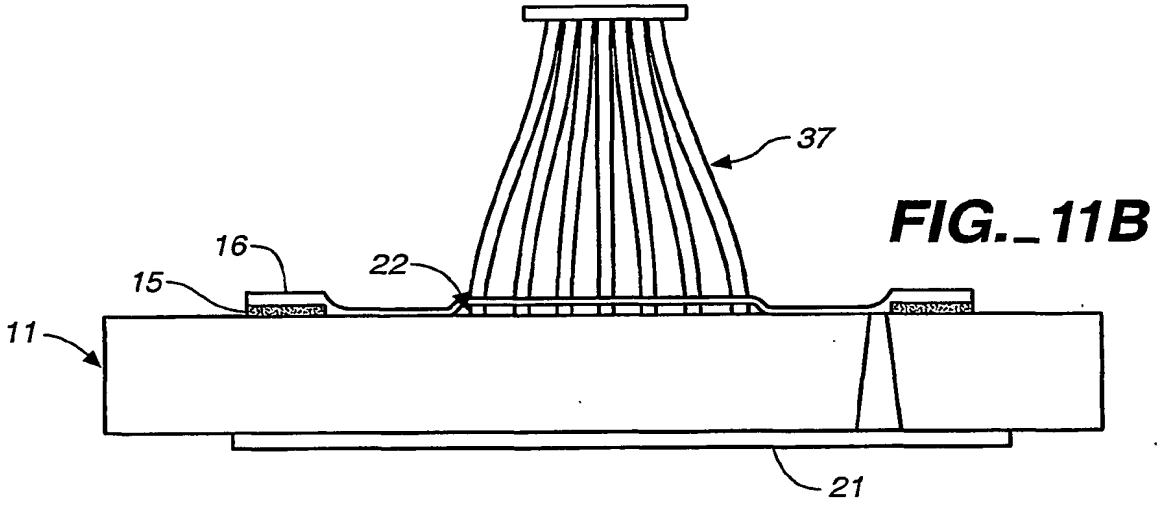


FIG._11B

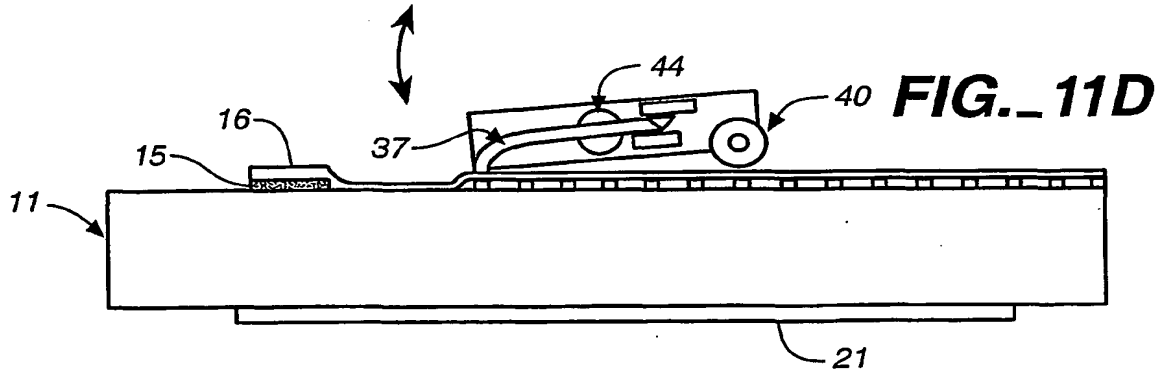


FIG._11D

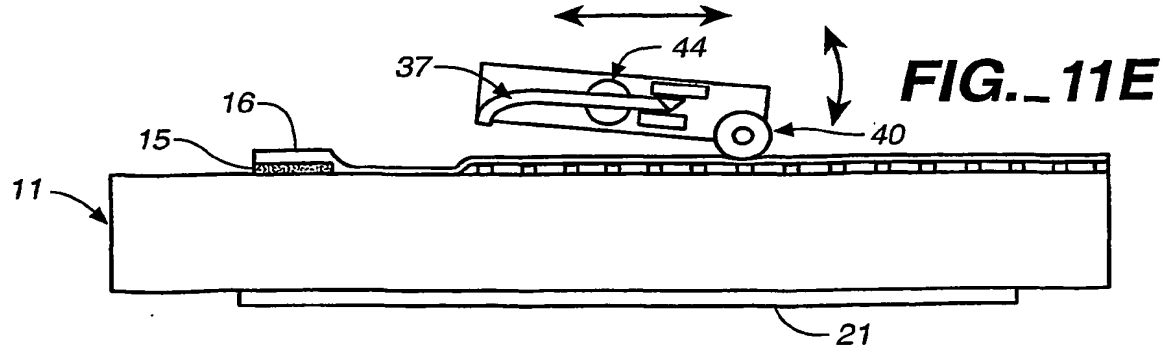


FIG._11E

FIG._12A

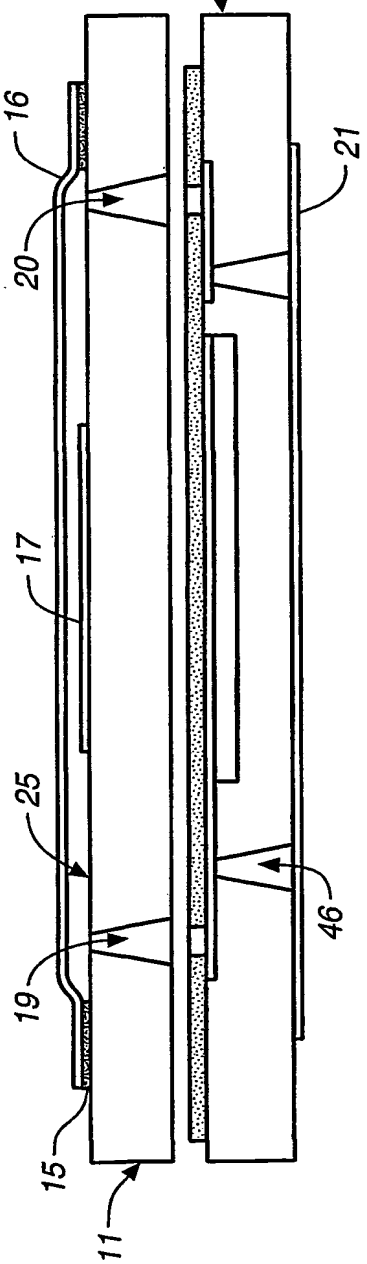


FIG._12B

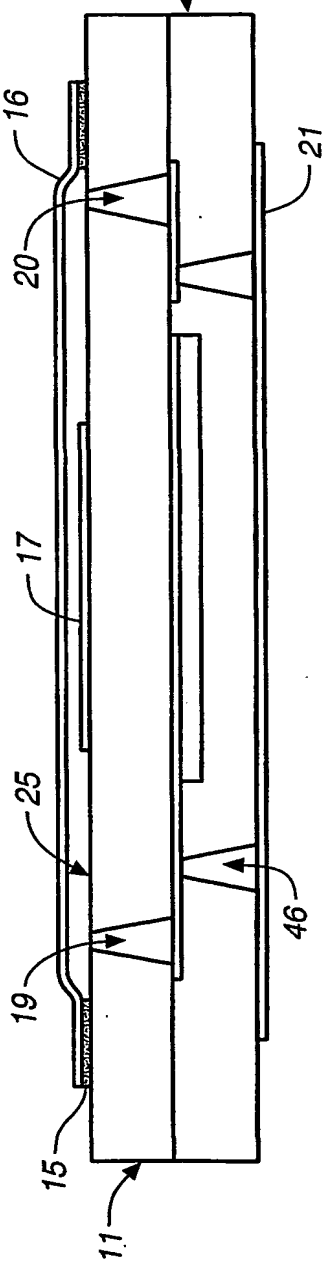


FIG._12C

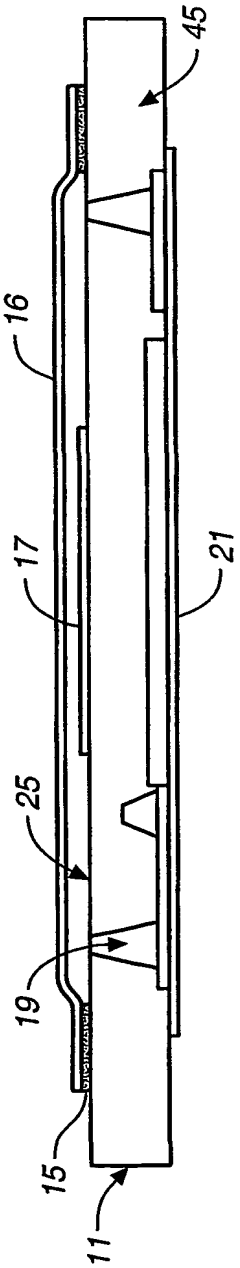
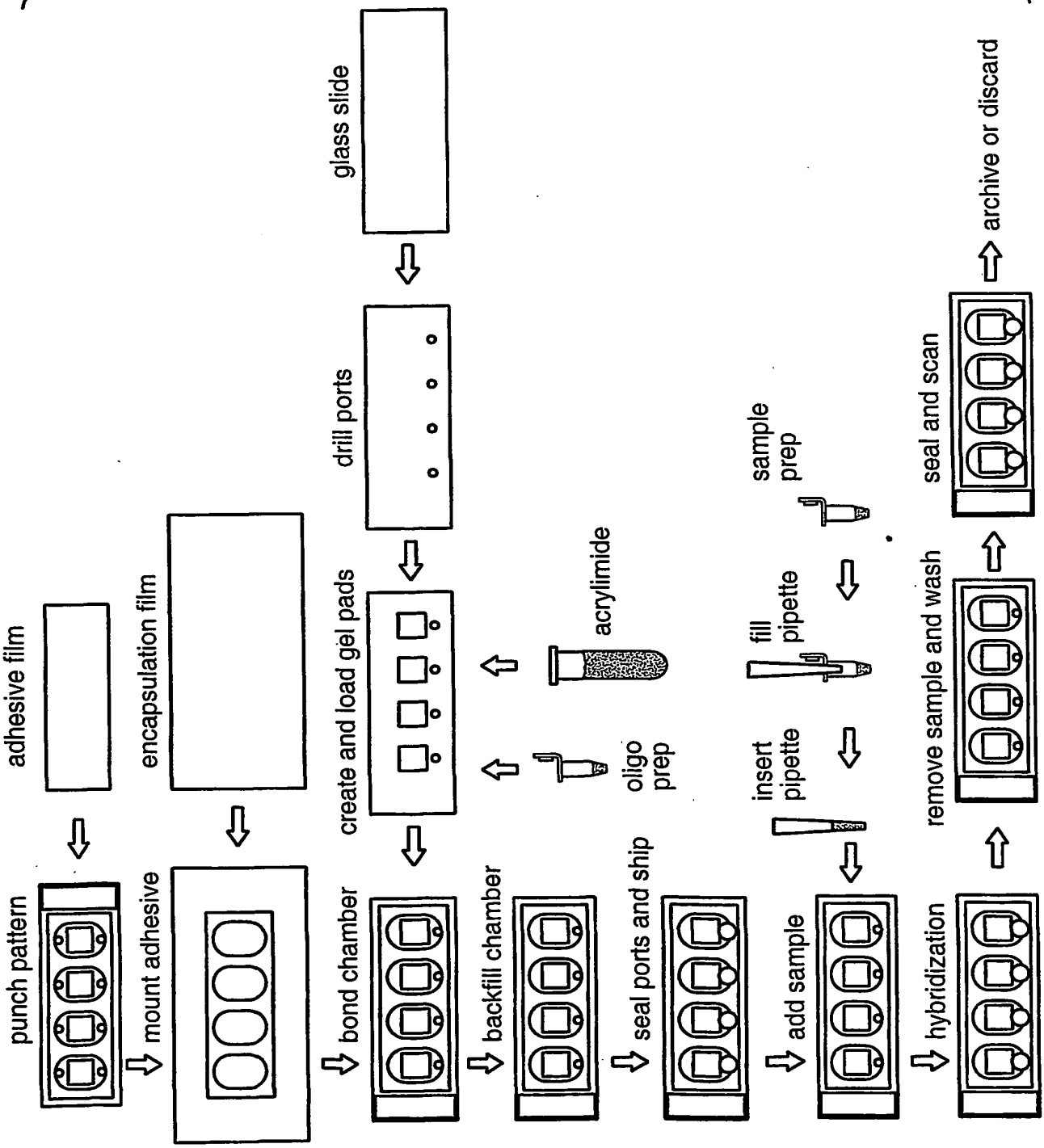


FIG._13



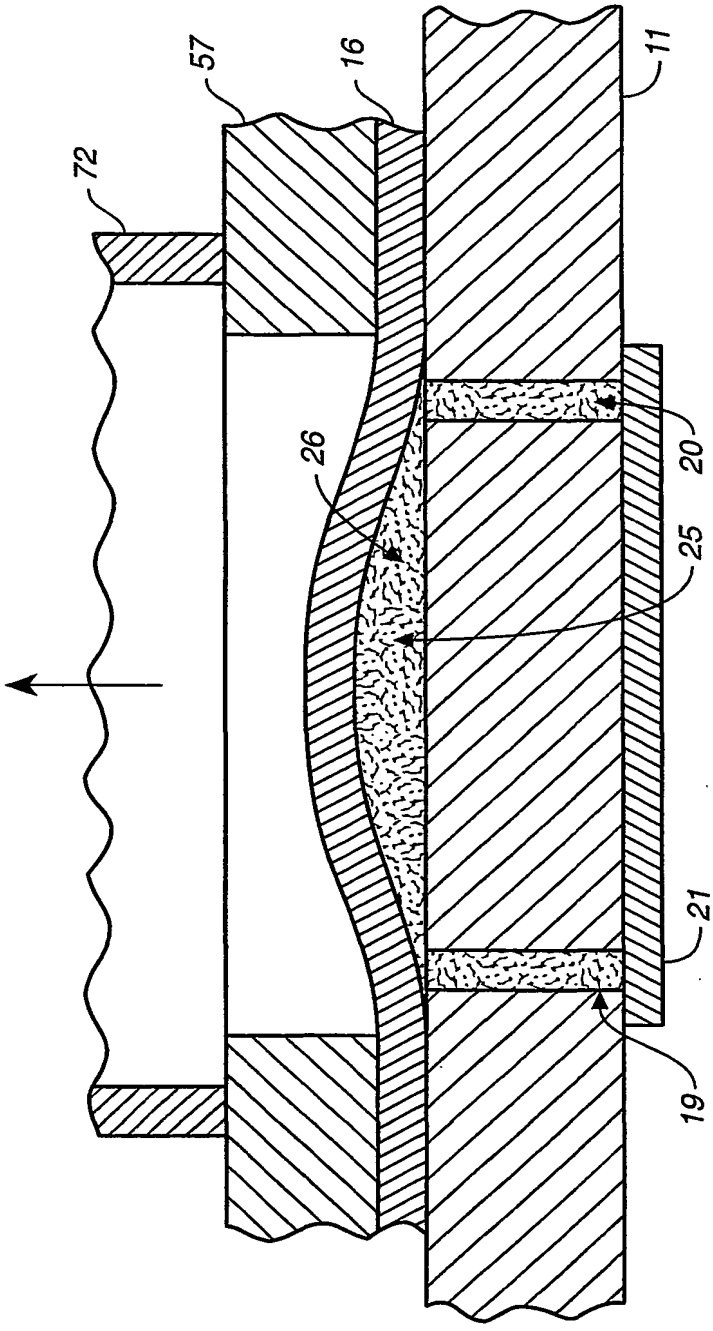


FIG._14

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 01/02664

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 B01L3/00 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 B01L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 922 591 A (FODOR STEPHEN P A ET AL) 13 July 1999 (1999-07-13) cited in the application column 2, line 29 - line 34 column 6, line 17 - line 24	1-5,8-11
X	column 8, line 51 - line 54	5
Y	column 29, line 47 -column 30, line 12; figure 12A column 32, line 30 - line 57; figure 7A	7
Y	column 35, line 25 - line 43	6
Y	column 37, line 4 - line 32	6
Y	US 3 429 796 A (LAUER JAY M) 25 February 1969 (1969-02-25) column 3, line 15 - line 20	7
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
29 August 2001	05/09/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Hocquet, A

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 01/02664

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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Y	US 5 798 215 A (RIBI HANS O ET AL) 25 August 1998 (1998-08-25) column 8, line 18 - line 44	6
A	EP 0 796 917 A (BECTON DICKINSON CO) 24 September 1997 (1997-09-24) page 2, line 6 - line 7 page 2, line 55 -page 3, line 22	6

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